PRACTICAL BLOOD GROUPING

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"I beg you to observe and to see the thing with your own eyes."

William Harvey.

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PREFACE

Those actively concerned in blood grouping work will have many methods which they favour. In writing this book our purpose has been to describe some of the methods which we have used, together with their pitfalls, in the hope that these observations may, in large or small part, be of value to others.

Many workers now have an interest in blood grouping, either in hospital laboratories, in other specialized laboratories, or as part of general biological work. A practical manual may either attempt to cover all possible methods or confine itself to choosing those which its authors favour, or with which they are most familiar. We decided on the latter course in the hope that this approach would be of more practical value.

Technical methods change as new discoveries replace the older ones. We do not expect that our suggestions will be immutable, since this would be the antithesis of progress which has constantly improved techniques over the last decade.

We have extensively consulted other books, including *Blood Groups and Transfusion*, by A. S. Wiener (1948), 3rd Ed.; *Fundamentals of Immunology*, by W. C. Boyd (1956), 3rd Ed.; *Principles of Bacteriology and Immunity*, by W. W. C. Topley and G. S. Wilson (Ed. by G. S. Wilson and A. A. Miles) (1955), 4th Ed.; *Blood Groups in Man*, by R. R. Race and R. Sanger (1954), 2nd Ed.; *Blood Transfusion in Clinical Medicine*, by P. L. Mollison (1956), 2nd Ed.; and *The Distribution of the Human Blood Groups*, by A. E. Mourant (1954). We are glad to be able to record our debt to these workers.

We are grateful to our many colleagues for permission to publish cases: to Dr. Edith Paterson, Dr. N. W. Preston and Dr. D. Riding for assistance with animal work; to Dr. R. Ollerenshaw for photographic help; to Mr. M. G. Bulmer for his invaluable help with the statistical sections, and to Dr. J. Shone for reading all and Dr. W. d'A. Maycock and Dr. A. Wardlaw for reading parts of the manuscript.

Our own staff have worked hard to help us to work out and record our methods; our Laboratory Staff, particularly Dr. B. Stone and Miss J. A. Hancock, and our Research Assistants have helped with the original work on many serological problems; and our secretaries and many others have given unflagging assistance. To them all we express our gratitude and particularly to Miss J. A. Hancock for her untiring efforts throughout the preparation of the book.

F. S.
P. H. R.

INTRODUCTION

For many years it had been appreciated that there were serological species differences between animals and Man. When the blood of Man and animals was mixed together erythrocyte agglutination occurred and when their sera were mixed, precipitation was sometimes seen. Blood transfusion experiments in which the blood of animals was infused into Man failed. Blood transfusion from one man to another was attempted and in about 50 per cent of cases was found to result in serious reactions or death to the recipient. It was in 1900 that Landsteiner discovered the ABO groups, and the explanation why these reactions had occurred was made clear.

This discovery was of fundamental importance since it represented for the first time an intra-species difference, a serologically demonstrable difference, between one human being and another. It was a long time before more blood groups were discovered and it was not until 1911 that the sub-groups of group A were described by von Dungern and Hirszfeld. Experiments were next conducted on the effect of injecting human red cells into animals and as a result of this work Landsteiner and Levine (1927) described the MN and P groups. The first stage of blood group detection had been made by using the antibodies of one human being to detect blood groups present in another; in the second stage animal sera were prepared by immunizing the animals with human red cells. In the meantime, a third method had been used for the detection of blood groups, and this was by using rare and naturally occurring human antibodies not usually of immune origin, for example anti-P. Sometimes animal sera were used which contained similar agglutinins and anti-P was originally referred to as "extra-agglutinin 1" (Landsteiner and Levine, 1929).

The fourth method for investigating the possibility of other human blood groups was that of injecting animal erythrocytes into animals, and then testing the antiserum against human red cells. In 1940 Landsteiner and Wiener, using an antiserum prepared by injecting monkey cells into rabbits, described the Rh blood group.

After 1940 there really begins a new era in blood grouping work because of the interest aroused by the discovery of the Rh group and its clinical significance and importance. One cannot at this point, therefore, fail to applaud all the pioneer work of Landsteiner and his many associates, and the more we hear from them of his technical skills the more we realize that the subtleties of technique which he used could not be and never have been published. Landsteiner and his associates were responsible for the discovery of all the major blood groups right up to the time of his death in 1943.

Since the discovery of the ABO groups, blood transfusion became more widely practised, particularly following the introduction of sodium citrate as an anticoagulant by Lewisohn (1915a, b). Nevertheless, in spite of the use of blood of the same ABO group a number of serious haemolytic transfusion reactions were reported in the literature from time to time. After the discovery of the Rh blood group, Wiener and Peters (1940), and Wiener (1941) described firstly four and later ten more cases of haemolytic transfusion reactions apparently not due to ABO incompatibility. In the cases investigated it was found that there were differences in the Rh group between the blood of the patient and that of the donor. There were all degrees of severity from mild reactions to uraemia and death. These were usually referred to as intra-group transfusion reactions. It was soon realized that the Rh group was of considerable importance in blood transfusion work and should always be taken into account.

Levine and Stetson in 1939 described a transfusion reaction in a parturient woman following the delivery of a macerated foetus. They suggested that the origin of an atypical agglutinin in the mother's blood was immunization of the mother by the baby. In some of the transfusion reactions previously referred to, it was found that on occasion the first transfusion would result in a reaction and all these cases occurred in intra-partum or post-partum patients. It was suggested that the source of the original stimulus was the foetus. Levine, Katzin and Burnham (1941), following this study of transfusion accidents, observed that atypical agglutinins were present in the blood of mothers who gave birth to children affected with erythroblastosis foetalis. They said that the destruction of red cells could be explained in terms of the iso-immunization theory. "One may assume that the agglutinins in the mother's circulation under certain conditions are capable of penetrating the placental barrier so that these antibodies by their continual action on the blood cells and perhaps tissue cells of the foetus induce erythroblastosis foetalis and its several manifestations." Most of these agglutinins which they described had anti-Rh specificity. Burnham *et al.* (1941) remarked that erythroblastosis foetalis and transfusion accidents in pregnancy may have a common aetiology.

Thus the foundation stones were laid, firstly with the discovery of the Rh factor, secondly, following the study of intra-group transfusion reactions, which were shown often to be associated with pregnancy and the puerperium, and thirdly, with the observation that the children of mothers who had these transfusion reactions were often affected with some or all of the manifestations of erythroblastosis foetalis. Levine, Burnham, Katzin and Vogel (1941) further developed the theory of iso-immunization with the Rh factor to explain the aetiology of the disease.

Haemolytic disease of the newborn, or erythroblastosis foetalis, is a

haemolytic state of the child in the neo-natal period. Three types used to be recognized: 1. hydrops foetalis, 2. icterus gravis neonatorum, and 3. haemolytic anaemia of the newly-born.

We do not nowadays see these cases in their fully developed form as often as we did years ago, because of the development of antenatal testing and the treatment of these babies at birth. The general theory of iso-immunization suggests that the foetus immunizes the mother to an antigen which she lacks in her red blood cells but which it possesses itself, having inherited it from the father. The mother develops antibodies in her serum (most frequently, anti-Rh antibodies) which pass back through the placenta and damage the child's red cells and possibly its tissues, so that it suffers from a haemolytic anaemia and other manifestations of haemolytic disease.

The realization of this has given rise, throughout most of the world, to antenatal testing services in which the blood groups of the mother are determined in the antenatal period and her serum searched for Rh antibodies, and if possible for other atypical blood group antibodies. Those whose sera show the presence of these antibodies are admitted to hospital and preparations undertaken to treat the child at birth. This usually involves an exchange transfusion.

During the antenatal examination of the maternal sera, many sera of Rh-negative mothers were investigated and found to contain no Rh antibodies active as saline agglutinins, but the mothers were sometimes delivered of children quite severely affected with haemolytic disease of the newborn. No explanation of this was forthcoming until in 1944 Race and Wiener independently observed what are now known as incomplete Rh antibodies. The first test devised for the detection of these was a blocking test, i.e. cells coated with incomplete antibody are not agglutinated by a saline agglutinating serum. Although this technique was an advance, it was by no means a satisfactory one. In 1945, Diamond and Denton introduced the use of 20 per cent bovine albumin for the detection of incomplete Rh antibodies. If bovine albumin was not available, human plasma, so selected as to lack rouleaux forming properties, was recommended. In 1945, Coombs, Mourant and Race described the test now known as the Coombs or antiglobulin test, which has been a major step forward in technical progress. It enabled incomplete blood group antibodies of many specificities to be detected and has been a most valuable test. Morton and Pickles (1947) demonstrated that trypsinized red cells were agglutinable by incomplete anti-Rh sera in saline suspension, whereas normal red cells were not.

Here, therefore, we had great technical advances in the space of a few years, greater than that made in the forty years between the discovery of the ABO and Rh blood groups. It had its reward and many new blood groups were discovered in quick succession.

The discovery of so many new blood groups would not have occurred, even with technical advances, or perhaps the technical advances themselves would not have occurred, if there had not been awakened a very great interest in blood and its serological properties and reactions, by the establishment during the war, in Britain and overseas, of Blood Transfusion Services. The demand for blood grew, and has steadily increased ever since as a result of the increasing complexity of medical and surgical procedures. The result of this has been that many thousands of blood samples have been tested in many ways and the sera of many persons have been examined; many new blood groups have been established and the complexity of others has greatly increased with the passage of time. The establishment of antenatal testing services, often as a part of the Blood Transfusion Services, also resulted in the examination of large numbers of blood samples. If unexpected or anomalous results occurred these were closely investigated. When cases of erythroblastosis foetalis, or haemolytic disease, arose in Rh positive mothers, atypical antibodies were discovered and characterized in their sera. These were occasionally found to react with hitherto unknown antigens on the human red cells, or with antigens that were part of an already established blood group system.

As a result of the establishment of these services, the blood of very large numbers of people were tested not only in the U.K. but throughout the world, and the frequency of various blood groups was found to be different in different populations. Sometimes the difference was not a great one but a gradual trend; for example, the difference in the ABO blood group frequencies throughout this country. In some cases, however, new blood groups were found in which there were marked frequency differences as between one population and another. Mourant has summarized these (1954) and indeed quotes 1,716 references in his book on the distribution of the human blood groups. From a practical point of view, it is often of importance to know with what population one is dealing, because technical requirements may be different between one population and another. For example, where Rh-negative individuals are rare in a population, as in the Chinese and Burmese, there is hardly any point in Rh testing of persons. In populations which contain a high percentage of Negroes, as in the United States of America, more extensive tests for the D^u group of Rh have to be conducted than those that are satisfactory for the British population. Moreover, certain blood group antigens may be more or less confined to a particular ethnic group; for example, the V group of Rh (DeNatale, Cahan, Jack, Race and Sanger, 1955) is pretty well confined to Negroes. The Diego blood group antigen (Layrisse *et al.*, 1955) is also most frequently encountered in the blood of certain South American Indians and is only very exceptionally met with in Caucasian populations. It is evident, therefore, that the

distribution of blood groups in different parts of the world is a matter of considerable practical importance.

Soon after the discovery of the first blood groups it was found that they were inherited. The presence of the antigen on the red cells was found to be determined by a corresponding gene or genes, and the study of blood group inheritance has been very considerable.

In these investigations the first step is the determination of the frequencies of the various blood groups in the population. From these phenotype frequencies it is possible to determine gene frequencies by calculation. Family studies are next made and the members of the family grouped for the particular blood group under investigation. These are now analysed and the observed frequencies of each type of mating, and of the children born from that mating, are compared with those that one would expect to occur; these latter being calculated from the gene frequencies previously obtained. Most blood groups are dominant characters, but in the Lewis group the mating Le(a −)× Le(a −) was found to give Le(a+) children (Andresen, 1948) and it is considered that this group is inherited as a recessive character.

The blood groups are, in many ways, ideal characters from the point of view of inheritance studies. They are normal physiological characters and as such are a feature of the whole population. Although in some cases technically difficult to determine, they are nevertheless, from a genetical point of view, clear-cut characters which can be charted and scored, and their manner of inheritance is largely simple, though it is now known that some of them, such as the ABO blood groups, may be affected by modifying genes (Weiner *et al.*, 1957) and even possibly by disease.

All human genetical studies usually involve the blood groups, particularly those involving determination of linkage. The various blood group systems are inherited independently of sex and that other useful character, taste-blindness. At one time it was thought they were all inherited independently of each other, but now it is known (Mohr, 1951a and b) that the Lutheran and Lewis blood group genes are linked or rather the Lutheran and secretor blood group genes are linked (Race and Sanger, 1958). This is one of three linkages that have been established between blood groups and other human characters. The other two are between the Rh groups and ovalocytosis, i.e. oval-shaped erythrocytes (Chalmers and Lawler, 1953), and that between the ABO group and the nail-patella syndrome (Renwick and Lawler, 1955).

Various other linkages have been described, notably that between the gene for sickle cells and the MN genes (Snyder, Russell and Graham, 1947), but this was disproved by Waller, Waller and Hughes (1952). Many other studies that have been made to determine linkage between human characters and blood groups have all failed.

Human twin studies have been extensive and blood groups are of

undoubted importance here. Clearly, twins are dizygous if they are of different sexes, or if their blood groups are different, but if their blood groups are the same they may still, of course, be dizygous, but they may also be monozygous and the probability of monozygosity can be calculated (Smith and Penrose, 1955). Stratton (1953) described a number of twin studies in which the twins were thought to be identical but one possessed a congenital abnormality that the other did not; calculations of the probability of monozygosity were made in these cases.

Stratton, Renton and Hancock (1958) recently summarized three ways in which disease might affect red cell agglutinability. These are:

1. Changes that occur in the ABO groups of red cells in certain diseases. Van Loghem *et al.* (1957) have previously published a case in which the ABO blood group of a patient appeared to have changed during the course of the disease, and the authors described a similar case.
2. They referred to polyagglutinability, which is a change produced in the red cells by disease, and described a case in which it was thought that disease was also responsible for the presence of a pan-agglutinin (anti-h) in the serum (Davidsohn and Toharsky, 1940).
3. A further way in which disease can affect the agglutinability of red cells was that described by Bessis *et al.* (1954), in which they showed that in sickle cell anaemia during the sickling stage the cells were less agglutinable.

Many studies have been made to determine any possible relationship between blood groups and disease. The most clear cut of all, of course, is that previously referred to, of haemolytic disease of the newborn. In 1953, Aird, Bentall and Fraser-Roberts published evidence that the frequency of the blood group A is greater and the frequency of blood group O less in patients suffering from gastric carcinoma than those in the general population of the same area. The evidence was based on cases of carcinoma collected from England and Scotland. Since that time a great deal more work has been done in an endeavour to find diseases in which the patients have different blood group frequencies from the normal population, and some contradictory results have been obtained, and of course the question of control groups is very important in this respect.

Blood groups have long been used for medico-legal work, and since there are nine blood group systems and many complications of each the number of possible blood group combinations runs into many hundreds of thousands. Landsteiner looked forward to the day when the blood groups would distinguish one person from another, just as fingerprints do at the present time. We are certainly moving towards that era.

In medico-legal work blood groups have been used to help to solve problems of identity and cases of disputed parentage. Cases of disputed parentage are most frequently concerned with disputed paternity. The ABO, MN and Rh systems are used and the combined chances of exclusion based on these three blood groups is 50 per cent.

When the newer blood groups came to be studied, particularly the Rh group, alternative theories were put forward to explain the various results obtained. We are concerned in this book with practical blood grouping, and our results would be the same in any language. We, therefore, feel absolved from commenting in detail on the various merits and demerits of systems of nomenclature and theories of inheritance in respect of the Rh and other blood groups.

It will have been observed from this short summary that the blood groups now are of considerable interest to many workers. They are of interest to clinicians, to pathologists and haematologists, they are of interest to biologists and geneticists and to anthropologists and even, indeed, to statisticians. This is why practical blood grouping is of general and wide interest.

References

Aird, I., Bentall, H. H., and Fraser Roberts, J. A. (1953). A relationship between cancer of stomach and the ABO blood groups. *Brit. Med. J.*, i, 799.

Andresen, P. H. (1948). The blood group system L. A new blood group L_2. A case of epistasy within the blood groups. *Acta Path. Microbiol. Scand.*, **25**, 728.

Bessis, M., Bricka, M., Breton-Gorius, J., and Tabuis, J. (1954). New observations on sickle cells, with special reference to their agglutinability. *Blood*, **9**, 39.

Burnham, L., and Engelwood, N. J. (1941). The common etiology of erythroblastosis and transfusion accidents in pregnancy. *Am. J. Obst. Gynec.*, **42**, 389.

Chalmers, J. N. M., and Lawler, Sylvia D. (1953). Data on linkage in man: elliptocytosis and blood groups. I. Families 1 and 2. *Ann. Eugen.*, **17**, 267.

Coombs, R. R. A., Mourant, A. E., and Race, R. R. (1945). A new test for the detection of weak and "incomplete" Rh agglutinins. *Brit. J. Exp. Path.*, **26**, 255.

Davidsohn, I., and Toharsky, B. (1940). The production of bacteriogenic hemagglutination. *J. Inf. Dis.*, **67**, 25.

De Natale, A., Cahan, A., Jack, J. A., Race, R. R., and Sanger, Ruth (1955). "V", a new Rh antigen, common in negroes, rare in white people. *J. Amer. Med. Ass.*, **159**, 247.

Diamond, L. K., and Denton, R. L. (1945). Rh agglutination in various media with particular reference to the value of albumin. *J. Lab. Clin. Med.*, 30, 821.

Dungern, E. v., and Hirszfeld, L. (1911). Uber gruppenspezifische strukturen des blutes III. *Z. Immun. Forsch.*, 8, 526.

Landsteiner, K. (1900). *Zur kenntnis der antifermentativen, lytischen und agglutinierenden wirkungen des blutserums und der lymphe. Zbl. Bakt.*, 27, 357.

Landsteiner, K., and Levine, P. (1927). Further observations on individual differences of human blood. *Proc. Soc. Exp. Biol. and Med.*, 24, 941.

Landsteiner, K., and Levine, P. (1929). On isoagglutinin reactions of human blood other than those defining the blood groups. *J. Immunol.*, 17, 1.

Landsteiner, K., and Wiener, A. S. (1940). An agglutinable factor in human blood recognized by immune sera for rhesus blood. *Proc. Soc. Exp. Biol. and Med.* 43, 223.

Layrisse, M., Arends, T., and Dominguez, Sisco R. (1955). Nuevo grupo sanguineo encontrado en descendientes de Indios. *Separata de Acta Med. Venezolana*, 3, 132.

Levine, P., and Stetson, R. E. (1939). An unusual case of intragroup agglutination. *J. Amer. Med. Ass.*, 113, 126.

Levine, P., Burnham, L., Katzin, E. M., and Vogel, P. (1941). The role of isoimmunisation in the pathenogenesis of erythroblastosis fetalis. *Am. J. Obst. Gynec.*, 42, 925.

Levine, P., Katzin, E. M., and Burnham, L. (1941). Isoimmunization in pregnancy, its possible bearing on the etiology of erythroblastosis fetalis. *J. Amer. Med. Ass.*, 116, 825.

Lewisohn, R. (1915a). A new and greatly simplified method of blood transfusion. *Med. Rec.*, 87, 141.

Lewisohn, R. (1915b). Blood transfusion by the citrate method. *Surg. Gyn. and Obstet.*, 21, 37.

Loghem, J. J. van, Dorfmeier, Hanny, and Hart, Mia van der (1957). Two A antigens with abnormal serologic properties. *Vox Sang.*, 2, 16.

Mohr, J. (1951a). A search for linkage between the Lutheran blood group and other hereditary characters. *Acta Path. Microbiol. Scand.*, 28, 207.

Mohr, J. (1951b). Estimation of linkage between the Lutheran and the Lewis blood groups. *Acta Path. Microbiol. Scand.*, 29, 339.

Morton, J. A., and Pickles, Margaret M. (1947). Use of trypsin in the detection of incomplete anti-Rh antibodies. *Nature*, 159, 779.

Mourant, A. E. (1954). *The Distribution of the Human Blood Groups*. Oxford: Blackwell Scientific Publications.

Race, R. R. (1944). An "incomplete" antibody in human serum. *Nature*, 153, 771.

Race, R. R., and Sanger, Ruth (1958). The Lutheran-secretor linkage in man: support for Mohr's findings. *Heredity*, in the press.

Renwick, J. H., and Lawler, Sylvia D. (1955). Genetical linkage between the ABO and nail-patella loci. *Annals Hum. Genet.*, 19, 312.

Smith, Sheila M., and Penrose, L. S. (1955). Monozygotic and dizygotic twin diagnosis. *Annals Hum. Genet.*, 19, 273.

Snyder, L. H., Russell, H., and Graham, Eugenia B. (1947). Linkage between the genes for sickle cells and the M-N blood groups. *Science*, 106, 347.

Stratton, F. (1953). *Clinical Genetics*, chap. iii. Ed. Arnold Sorsby. London: Butterworth and Co. (Publishers) Ltd.

Stratton, F., Renton, P. H., and Hancock, Jeanne A. (1958). Red cell agglutinability affected by disease. *Nature*, 181, 62.

Waller, Marion, Waller, R. K., and Hughes, R. D. (1952) Linkage studies of genes for sickling and MNS blood groups: negative findings. *Proc. Soc. Exp. Biol. and med.*, 80, 479.

Weiner, W., Lewis, H. B. M., Moores, Phyllis, Sanger, Ruth, Race, R. R. (1957). A gene, y, modifying the blood group antigen A. *Vox Sang.*, 2, 25.

Wiener, A. S. (1944). A new test (blocking test) for Rh sensitization. *Proc. Soc. Exp. Biol. and med.*, 56, 173.

Wiener, A. S. (1941). Hemolytic reactions following transfusions of blood of the homologous group. II. Further observations on the role of property Rh, particularly in cases without demonstrable isoantibodies. *Arch. Path.*, 32, 227.

Wiener, A. S., and Peters, H. R. (1940). Hemolytic reactions following transfusions of blood of the homologous group, with three cases in which the same agglutinogen was responsible. *Ann. Int. Med.*, 13, 2306.

CHAPTER 1

ANTIGEN-ANTIBODY REACTIONS

Antigens

An antigen is a substance which, when injected parenterally into an animal, causes the production of an antibody which reacts specifically with that antigen in some observable manner. An antibody, conversely, is a protein present in the blood serum which is often produced as the result of an antigenic stimulus and which reacts with the antigen in some observable manner. Antigens and antibodies react together specifically (although not absolutely specifically, as will be seen later) to give an observable result by which it is known that this antigen-antibody reaction has taken place, and the observable reaction will depend very largely upon the state of the antigen or the nature of the antigen bearing organism or red cell. In the case of red blood cell antigens and their corresponding antibodies the observable results are agglutination or haemolysis of the erythrocytes. The immediate effect of an antigen-antibody reaction may be to produce neither agglutination of the red cell nor its haemolysis, but subsequent treatment of the sensitized cells may eventually produce red cell agglutination which shows that originally an antigen-antibody reaction had taken place. That an antigen-antibody reaction has occurred in the absence of agglutination or haemolysis of the red cell may also be inferred if it can be shown that other serological changes have taken place which are commonly associated with antigen-antibody reaction—for example, the absorption or fixation of complement.

Blood groups are represented by antigens present on the surface of red blood cells, and the antibodies to these antigens are the blood group antibodies. Many of the features normally associated with antigen-antibody reactions are known to be appropriate to blood group antigen-antibody reactions and it is, therefore, interesting to know more of the general phenomenon itself. Antigens are complex chemical substances, generally of a protein nature, although many non-protein antigens are known. Sometimes the protein may be very complex and may contain many different antigens.

Landsteiner (1945) introduced the term "hapten" to describe "specific protein-free substances which although reactive in vitro induced no, or only slight, antibody response". These partial antigens, sometimes polysaccharides, may combine with the antibody in some observable way. The A and B antigens of human red cells are examples of muco-polysaccharides. Aminoff, Morgan and Watkins (1950) isolated group A substance from human ovarian cyst fluid; it had a molecular weight of 260,000. These purified polysaccharides, if of A

Snyder, L. H., Russell, H., and Graham, Eugenia B. (1947). Linkage between the genes for sickle cells and the M-N blood groups. *Science*, **106**, 347.

Stratton, F. (1953). *Clinical Genetics*, chap. iii. Ed. Arnold Sorsby. London: Butterworth and Co. (Publishers) Ltd.

Stratton, F., Renton, P. H., and Hancock, Jeanne A. (1958). Red cell agglutinability affected by disease. *Nature*, **181**, 62.

Waller, Marion, Waller, R. K., and Hughes, R. D. (1952) Linkage studies of genes for sickling and MNS blood groups: negative findings. *Proc. Soc. Exp. Biol. and med.*, **80**, 479.

Weiner, W., Lewis, H. B. M., Moores, Phyllis, Sanger, Ruth, Race, R. R. (1957). A gene, y, modifying the blood group antigen A. *Vox Sang.*, **2**, 25.

Wiener, A. S. (1944). A new test (blocking test) for Rh sensitization. *Proc. Soc. Exp. Biol. and med.*, **56**, 173.

Wiener, A. S. (1941). Hemolytic reactions following transfusions of blood of the homologous group. II. Further observations on the role of property Rh, particularly in cases without demonstrable isoantibodies. *Arch. Path.*, **32**, 227.

Wiener, A. S., and Peters, H. R. (1940). Hemolytic reactions following transfusions of blood of the homologous group, with three cases in which the same agglutinogen was responsible. *Ann. Int. Med.*, **13**, 2306.

homologous antibody is lost. The activity of certain blood group antigens is reduced by heating at 56° C. for even a short period of time, (Murray and Clark 1952).

In the definition of an antigen it has been stated that it combines with antibodies specifically, but the nature of this specificity needs further consideration. Specificity depends on chemical structure. The classical work of Landsteiner and his colleagues showed that this specificity was not an absolute one. Antibodies combine most strongly with homologous antigen, but will cross react with antigens whose chemical structure is closely related to it. It is not easy to demonstrate the dependence of serological specificity on chemical structure when using complex antigens such as human serum. Where a single synthetic antigen, which can be chemically modified, is used, cross reactions can be closely studied. From the point of view of blood grouping the important point to appreciate is that the term "specific" with reference to antigen-antibody reactions has not an absolute meaning.

When considering the response of an animal to an antigenic stimulus the work of Medawar and his colleagues (Billingham *et al.*, 1956) shows that one must have regard to the nature of the antigenic stimuli to which the animal has been subjected during its foetal life. These workers have shown that an adult animal will not respond to an antigen to which it was exposed during foetal life. In humans this is well illustrated by the occurrence of human chimera, where, owing to an anastomosis between the circulation of fraternal twins in utero, cells migrate from one twin and lodge in the marrow of the other, where they proliferate. If the twins are of different blood groups a mixed blood can result with, for example, both A and O cells present. If the migrating cells are group A, and their recipient is group O, the latter fails to produce anti-A in the serum, although anti-B is produced normally (Dunsford *et al.*, 1953; Nicholas *et al.*, 1957; Booth *et al.*, 1957). The phenomenon is common in cattle but rare in humans and is referred to by Billingham *et al.*, as "actively acquired tolerance".

Erythrocytes are complex structures containing many antigenic components of different specificities. These are characterized, among other things, as the various blood groups. Consequently, if erythrocytes are used as antigens, either in Man or animals, many different antibodies may be produced and these may be separated from one another by absorption and various other means.

Antibodies

Antibodies can be described in many ways. They may be designated in terms of the antigen which is used to produce them, that is, the homologous antigen, or in terms of the animal in which they appear. For example, an antibody to human group A red cells made in rabbits is referred to as "rabbit-anti-A antibody", and if it occurs in humans,

specificity, will produce a precipitate when mixed with suitable anti-A sera. Witebsky, Klendshoj and McNeil (1944) showed that purified polysaccharides would produce high titre anti-A and anti-B when injected into humans; about half respond well. Kabat, Baer, Day and Kraut (1950) showed that human A substance would provoke an antibody response in a Group O individual.

The proteins of animals of different species are serologically different. This is known as species specificity and the further apart animals are on the biological scale, the easier it is to demonstrate. With certain proteins, such as globulins, it is readily demonstrable, but with others, such as albumin, it is more difficult. Nevertheless, Dakin and Dale (1919) were able to distinguish serologically between duck and hen albumin. The distinction between the serum of Man and lower vertebrates is easily made, that between Man and monkeys is more difficult, and that between Man and chimpanzees most difficult, and considerable cross reactions occur.

It is sometimes found that an antigen from one species will produce antibodies which cross react with antigens from an unrelated species. Antibodies prepared against a particular antigen, such as guinea-pig kidney, may be found to haemolyse sheep red cells or react with bacteria or other animal tissue. This is the Forssman antigen or hapten. These antigens are often referred to as heterogenetic antigens and cross reactions have been described (Boyd, 1939) between a particular antigen and antigenic groupings which are dispersed throughout nature without regard to species. The A and B blood group antigens are heterogenetic antigens. The Forssman antigen itself is present in the red cells of persons who are group A and AB.

What makes a substance antigenic in a particular animal is not accurately known. Antigens are substances foreign to the animal and of certain molecular size and structure. Antigens are effective in some animals and not in others, and the host must always be considered when referring to the antigenicity of a particular substance.

The blood group antigens vary in this respect as do other antigens. For example, although A and B are antigenic in Man, they are antigenic in animals as well, and good antibodies can be produced in rabbits by the injection of red cells containing these substances. The D antigen, on the other hand, is a very good antigen in Man, but it is only with [illegible]
[illegible]
[illegible]
antigenic in Man. On the contrary, the MN antigens of the red blood cells are very antigenic in rabbits and high titre antibodies can be produced, but when injected into Man antibodies to these antigens are almost impossible to produce. Antigenicity may be present in a denatured, or partially denatured, protein, but their ability to react with

reference to anti-D on page 174, and it is interesting to notice in the present connection that the agglutinating and incomplete preparations of colloidal silica show the same effects. This is illustrated by Table 1, which should be compared with Table 51 (p. 174), where the effect given by antibodies is seen.

Chemically, antibodies are a particular type of serum globulin, largely γ globulin, and antibody globulins have about the same molecular weight as the normal serum globulins in the fraction in which they occur, at least in Man and rabbits (Boyd, 1956); for example, γ globulins of molecular weight of the order of 100,000–150,000. The qualitative nature of the change that takes place in a normal serum globulin and confers on it the particular characters that make it an antibody globulin is unknown, but quantitatively there may occur, following immunization, an increase in the serum globulin of the animal. Antibodies may be γ globulins or α and β globulins and, apart from their immunological properties, are indistinguishable from the normal serum globulins of the animal. In some animals, such as the horse, immunization produces a new electrophoretic component. The serum component in which the antibody resides depends to some extent on the nature of the antigen and the degree to which the animal has been immunized. Prolonged immunization may result in antibody β globulins being produced.

Purification of antibody can be effected in two ways. Firstly, by physical and chemical means (e.g. Cohn's alcohol precipitation technique, where the γ globulins can be separated from the other proteins); secondly, by elution of the absorbed antibody from the antigen substrate (p. 26). Blood group antibodies can be purified by either of these methods.

The antibodies that occur in animal sera are divided into two kinds, natural and immune.

Natural antibodies might be defined as a property of the serum protein itself. Species specific antibodies are often thought to belong to this category. It has been suggested by Boyd and Bernard (1937) that all γ globulin might be antibody, so that if the serum of Man or animals were to be exhausted with all the antigens one could find, there would be a very substantial reduction in the serum globulin of the animal. It has been contended that iso-antibodies, such as anti-A and anti-B, which occur in varying strengths in human sera, are of natur[illegible] *al.*, 1953) [illegible] ens with [illegible] be borne [illegible]. During life one will constantly be immunized by such antigens without obviously having received them by way of blood transfusion or other means (Wiener, 1951).

Immune antibodies are those produced as a result of parenteral

as a "human anti-A antibody", and so on. On the other hand, they may be described with reference to the nature of the observed reaction when the antigen and antibody are mixed together. For example, if agglutination is observed they are referred to as "agglutinins"; if haemolysis is observed, as "haemolysins". With organisms carrying antigens, such as bacteria, the death of the organism may result from an antigen-antibody reaction and the antibody be called "a bacteriocidin". The question arises as to whether all these different results imply that different antibodies are produced by the injection of a single antigenic component, or whether one antibody is produced which produces different effects according to different physical or chemical or other conditions when it reacts with the antigen. The unitarian hypothesis of the nature of antibodies is favoured by many immunologists, and indeed the same antibody might react with the antigen to produce different observable results. Nevertheless, an antigen may give rise to antibody molecules of differing activities and with various degrees of avidity towards the antigen.

Some antibodies, when they react with the antigen, may produce an observable result of one kind but not of another; for example, they may act as a haemolysin but not cause agglutination of the red cells. On some occasions antibodies unite with red cell antigens, but the fact that this has occurred is not apparent because agglutination does not occur in saline solutions but only in viscous media, or when the antiglobulin test is employed, or when the red cells are treated with enzymes. These antibodies are sometimes referred to as "incomplete" or "univalent" antibodies. Such antibodies commonly occur among blood group antibodies.

The exact physical or chemical properties which determine the difference between an agglutinin and an incomplete antibody are not known, though it has been suggested that incomplete antibodies are univalent and are smaller in size than agglutinins which are bivalent or polyvalent. Whether or not this explanation is correct, it seems that the difference must be something of a fairly simple nature, since many of the properties to which the interaction of incomplete antibodies and agglutinins give rise, such as blocking and zoning, can be mimicked by preparations of colloidal silica (Renton and Hancock, 1957). Some of these preparations resemble agglutinins and some resemble incomplete antibodies, and, by mixing the two types of preparation, phenomena similar to those observed with antibodies can be produced. When the proportion of incomplete antibody in a serum or mixture of sera is increased, whilst the amount of agglutinin remains constant, a progressively increasing prozone develops which eventually meets the titration end-point, so that the serum no longer agglutinates the cells at any dilution. The addition of the incomplete antibody does not affect the agglutinin titre. These phenomena are described with

introduction of antigen. It may be that immunization will occur in an animal that already possesses antibodies for that particular antigen.

Most blood group antibodies are of human origin, and the study of human proteins, therefore, is one that is most important in blood group work. Heating of human serum containing antibodies slowly decreases the activity of the antibodies and finally destroys them altogether, but the degree of heat that is required to destroy the activity of an antibody depends upon its nature. Incomplete antibodies seem to be more heat resistant than saline agglutinins.

Antigen-Antibody Reactions

Antigen-antibody reactions take place in two stages. In the first stage the antigen is combining with the antibody, and in the second stage further changes are taking place as a result of this union which make its effects manifest by agglutination or other means. The conditions which are optimal for the first stage are not necessarily the same as the conditions which are optimal for the second stage. This is well seen, for example, in the Donath-Landsteiner test, where the first stage only takes place at a low temperature, but the second stage, which consists of the lysis of the red cells, only takes place at 37° C. Thus the first stage produces no visible effect and it takes place very rapidly, whereas the second stage takes place more slowly and the observable effect only occurs if the physical and chemical conditions are right. The two stages are also of importance in connection with the phenomenon of zoning.

In the serum albumin test used to detect blood group antibodies there is evidence to suggest that the conditions for sensitization of the erythrocytes by antibody are different from those required for their agglutination. If these differences can be more accurately defined improvements may be made in the technique of this test.

Blood group antigen-antibody reactions are usually shown by agglutination in the second stage, and after the cells have been sensitized in the first stage, various special conditions are sometimes needed [illegible]

[illegible] serum, or it may be necessary to wash them and add an antiglobulin reagent in order to produce agglutination. It is generally thought that the lattice hypothesis of the action of the antibody is correct, at any rate where agglutination is concerned; that is to say, the agglutination is not brought about by the agglutinin merely altering the physical surface of the cells so that they are no longer able to remain in stable suspension, but by [illegible]
bridge between one red cell and ano[illegible]
the agglutinin molecule is attached [illegible]

TABLE 1. EFFECT OF "INCOMPLETE" COLLOID ON AGGLUTINATION

P	Dilutions of agglutinating colloid												
	256	512	1,000	2,000	4,000	8,000	16,000	32,000	64,000	128,000	256,000	512,000	1,000,000
0 to 1 ..	v	v	v	v	v	v	v	v	+++	++	w	—	—
128 to 1 ..	v	v	v	v	v	v	v	v	++	++	+	—	—
256 to 1 ..		+++	+++	v	v	v	v	v	++	+	+	w	—
512 to 1 ..			+	+	++	++	+++	v	++	+	+	—	—
1,000 to 1 ..				—	—	w	+	++	++	+	w	—	—
2,000 to 1 ..					—	—	—	+	w	+	w	—	—
4,000 to 1 ..						—	—	—	—	—	—	—	—
8,000 to 1 ..							—	—	—	—	—	—	—

Each test consisted of 1 vol. of agglutinating colloid, 1 vol. of "incomplete" colloid, and 2 vols. of cell suspension.

Each column shows results for a constant *dilution* of agglutinating colloid.

Each row shows results for a constant *proportion* of "incomplete" to agglutinating colloid, e.g. for a tube containing "incomplete" colloid diluted 1 in 2 and agglutinating colloid diluted 1 in 4,000. P = 2,000 to 1.

v, +++, ++, +, w = degrees of agglutination

— = *no agglutination.*

TABLE 2. ESTIMATION OF NUMBER OF MOLECULES INVOLVED IN AN ANTIGEN-ANTIBODY REACTION

(i) *Comparison of inhibitory effect of eluate and γ globulin on agglutination of Rh sensitised cells by anti-γ globulin reagent.*

Inhibitor	*Dilution of Inhibitor*					
	1	2	4	8	16	32
Eluate	–	w	++	+++	++++	++++
γ globulin 10×10^{-8} gm./ml.	–	–	–	++	+++	++++

∴ Eluate is equivalent to 5×10^{-8} gm./ml. of γ globulin.
Since molecular weight of γ globulin = 153,000
and weight H atom = $1{\cdot}66 \times 10^{-24}$ gm.
∴ Eluate contains $1{\cdot}97 \times 10^{11}$ molecules/ml.

(ii) *Titration of eluate with D positive cells.*
1 volume eluate to 1 volume cell suspension ($0{\cdot}91 \times 10^{7}$ RBC/ml.).

Method	*Dilution of eluate*				
	8	16	32	64	128
Antiglobulin test	+++	++	–	–	–
Papain cell test	++++	+++	++	+	–

∴ ++ antiglobulin test = 1,300 molecules/R.B.C.
and + papain cell test = 325 molecules/R.B.C.
A second determination gave:
++ antiglobulin test = 3,000 molecules/R.B.C.

Zoning is a phenomenon in which the agglutination, precipitation or other sign that an antigen-antibody reaction has taken place fails to occur with low dilutions of the antibody, but does occur with higher dilutions. The same phenomenon can occur in certain systems during titrations of antigen against a fixed quantity of antibody. Zoning from antigen excess is of little importance in blood group serology, but zoning from antibody excess is not uncommon. The first part of the titration, where the results are negative, or weaker than those in the later part of the titration, is called the prozone. The cause of this phenomenon, in agglutination systems at any rate, appears to be that [illegible] the second cell. The conclusions drawn from some studies on zoning with anti-D sera are discussed on p. 172.

to a second, and it will be seen that the idea that the agglutinin is bivalent or polyvalent is implicit in this hypothesis.

When an incomplete antibody and an antiglobulin reagent act together in bringing about agglutination, this will only take place if the two molecules involved are attached to the red cells in the correct order. This can be demonstrated by an experiment using an eluate of incomplete anti-D made from sensitized cells which have been thoroughly washed to free them from serum proteins before elution takes place. If this is done with care, the eluate will consist virtually of pure antibody, or, at any rate, the only substance in it capable of reacting with antiglobulin reagents is antibody. This can be shown by the fact that absorption of the eluate with thoroughly washed D-positive cells removes its ability to neutralise antiglobulin reagents. If such an eluate is mixed on a microscope slide with a 5 per cent suspension of thoroughly washed D-positive cells, the mixture left for five minutes and a volume of antiglobulin reagent added, the cells will be found to agglutinate and it appears that the cells first take up anti-D, and later the sensitized cells take up the antiglobulin reagent and agglutinate. If, however, the antiglobulin reagent and eluate are mixed together first, and the cells added five minutes later, no agglutination occurs. It seems from this that the anti-D and the antiglobulin reagent have combined together in such a way that the former can no longer attach itself to the D-positive cells.

Such a pure anti-D eluate not only fails to react with D-positive cells when first mixed with antiglobulin reagent, but it will also neutralize the antiglobulin reagent, and if the ability of such an eluate to neutralize antiglobulin reagent is compared with the ability of solutions of γ globulin of known concentration to do the same, it is possible to estimate the amount of active protein in the eluate. If we assume that one molecule of anti-D reacts with antiglobulin in the same way as one molecule of γ globulin, it is possible to estimate the number of molecules of anti-D in the eluate, and by using the eluate to sensitize D-positive cell suspensions of known strength one can arrive at an idea of the number of molecules of antibody needed for the cells to give a positive antiglobulin test. The method is obviously somewhat approximate and a variety of controls is needed, but working on these lines we estimated that about 1,000 to 3,000 molecules of anti-D per red cell are needed for the cells to give a ++ agglutination in the Coombs test, though one of the eluates which we studied would agglutinate papainized D-positive cells at a dilution corresponding to about 325 molecules per red cell (Table 2).

Boursnell, Coombs and Rizk (1953) calculated that there were 5,500 D combining sites on an Rh positive erythrocyte. This is interesting to compare with the above figures. *Filitti-Wurmser et al.* (1954) estimated 5×10^5 B sites on a group B red cell.



PLATE I. Microdissection of an anti-A agglutinate Strands of lozenge-shaped cells connected by invisible filaments can be seen See p 10. ×270

When antibody reacts with antigen certain serum components which are neither antigen nor antibody may be necessary for the antigen-antibody reaction to occur, or may be absorbed during its occurrence, or may be necessary for its detection. Complement is one such substance, and reference will also be made to another substance unlike complement, present in some normal sera and in pathological sera, which reacts with antigen-antibody aggregates.

THE STRUCTURE OF AGGLUTINATES. The agglutination of red cells by agglutinating antibodies seems to be due to the adherence together of "myelin forms" (Bessis, 1955), and if a clump of agglutinated cells is forcibly pulled apart it can be seen that the cells are held together in the clump by a very elastic material. The appearances are similar with agglutinins of various specificities but are best demonstrated following the intense agglutination of group A cells by anti-A. Plate I shows the general appearance seen on the micro-dissection of such an agglutinate. The agglutinate is in process of being pulled apart by two needles, only one of which can be seen in the illustration. Chains of cells are visible close to the needle and the cells in these chains are distorted into a lozenge shape and held together by invisible filaments. Plate II shows four steps in the micro-dissection of a clump of A cells agglutinated by anti-A. In (*a*) and (*b*) several chains of cells retain their continuity, and in (*c*) all the chains but one have broken and the one chain which remains unbroken is stretched almost to its limit. A single lozenge shaped cell is visible, still attached by its filaments to the cells near the needle at one end of the chain and to the cells of the clump at the other end. In (*d*) the needle has been moved a little further from the clump and the filament has broken between the needle and the isolated cell. The filament between the cell and the clump contracted at this point and drew the cell back to the clump, so that the cell is no longer visible in the photograph. If the cells attached to the needle are examined it can be seen that the appearance of tension seen in (*a*), (*b*) and (*c*) is no longer visible in (*d*) when the chain has broken.

SPECIAL SERUM FACTORS

Complement is a property of serum which may either cause lysis of sensitized sheep cells or agglutination of red cells treated with antibody in the presence of conglutinin. A guinea-pig serum contains a typical haemolytic variety of complement and horse serum a conglutinating [illegible]

[illegible] which was the name [illegible] This substance was [illegible] [illegible]oated with antibody after the aggregate had absorbed complement. The phenomenon is

most conveniently shown using cells coated with bovine antibody and absorbed horse complement. Such coated erythrocytes are agglutinated by a serum containing conglutinin. Streng (1930) described immuno-conglutinins which appear in animal sera following a variety of different inocula. Coombs and Coombs (1953) suggest that immuno-conglutinin interacts with adsorbed complement.

Haemolytic complement, on the other hand, is a thermo-labile substance, and inactivated by heating normal serum at 56° C. for 25 minutes.

Serum complement consists of four components (Kabat and Mayer, 1948), C'1, C'2, C'3 and C'4, and the titre of whole complement (C') is dependent on the titre of the weakest component. In human serum this is C'2, which is also the most thermo-labile. C'1 is also thermo-labile, but C'3 and C'4 are more resistant to the effect of heat. Mayer *et al.* (1954) have shown that complement fixation and lysis of red cells proceeds in three stages. The first stage requires Ca^{++} and probably involves C'1 and C'4; the second stage requires Mg^{++} and C'2, and in the terminal phase C'3 actively results in lysis of the cells.

It has long been known that certain antigen-antibody reactions can be detected owing to the fact that when they occur complement is fixed; this fixation of complement can be detected by an indicator system consisting of sensitized sheep cells. This, of course, is another way by which antigen-antibody reactions can be detected *indirectly*. Lepow *et al.* (1956) have recently shown that C'1 is a proesterase which is converted into an esterase during antigen-antibody reactions, and it seems possible that it destroys other components of complement, C'2 and C'4, and complement is thus fixed.

Pillemer *et al.* (1954) described a new natural immune property of blood, properdin. Properdin, complement and Mg^{++} form the properdin system, a natural defence mechanism of blood. Properdin (P) is removed from serum when it is treated with zymosan (Z), a complex carbohydrate of yeast, at 15° C. A PZ complex is formed which at 37° C. inactivates the C'3 component of complement; this is the basis of the properdin assay.

Properdin plays no part in blood group reactions, but complement does, and this is described in Chapter III.

The Rose-Waaler test, in which use is made of sensitized sheep cells for [illegible]
ma[illegible]
dilu[illegible] suitable conditions with sensitized sheep cells, a high agglutination titre is recorded, whereas normal sera give a much lower titre. Many workers subsequently found that some normal human sera would, under certain circumstances, agglutinate Rh positive cells coated with incomplete Rh antibody (Milgrom *et al.* 1956, and Grubb 1956). Such properties are relatively uncommon among normal sera but



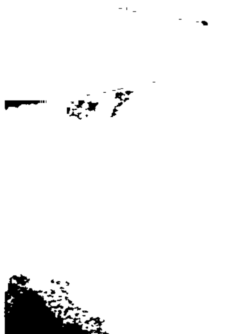
a



b



c



d

PLATE II. Four stages in the microdissection of an anti-A agglutinate. See p. 10. × 270

serum so that it is not detectable. This does not mean that the animal is as it was before the antigen was given, but a change has been produced so that a second dose of antigen at a later date will produce a much higher antibody response than the initial dose.

Blood group antigens are able to immunize human beings and this may arise in three ways:

1. By the injection of red cells, either accidentally, as in the case of blood transfusion, or purposely, with the object of stimulating antibody formation.
2. By the injection of specific mucopolysaccharides A and B.
3. As a result of pregnancy.

Antibodies against the A or B antigens are normally present in sera depending on the ABO group and the inoculation of them produces a marked rise in anti-A or anti-B titre. On the other hand, red cells containing antigens, such as the D antigen, may be injected against which antibodies are not normally present in human serum. Diamond (1944) injected Rh negative male volunteers with small quantities of Rh positive blood and three out of five were successfully immunized. Many other workers have experimented with this immunization process, sometimes giving or adding special adjuvants, as did van Loghem *et al.* (1949), who successfully produced anti-C^w antibody by injection of humans. Oftentimes it has been established that the injection of small doses of red cells containing the homologous antigen will boost the titre of an atypical antibody present in human serum. For example, the strength and titre of Rh antibodies are commonly increased in this way. This, of course, should not be done in mothers of the child-bearing years. Erythrocyte stroma is antigenic and Grove-Rasmussen *et al.* (1955) have recently described a case of an Rh negative person suffering from haemophilia in whom anti-Rh antibodies were induced by the injection of plasma due to the presence of red cell stroma in the plasma.

ISO-IMMUNIZATION DUE TO PREGNANCY. If a mother lacks a particular blood group factor in her red cells, she may be immunized during the course of pregnancy by this blood f[illegible]
baby's red cells, having be[illegible]
ization will produce in the [illegible]
antigen. The commonest antigen to which this occurs is the D antigen of the Rh series. In this particular instance the mother is Rh negative, the father is Rh positive, and the baby is Rh positive. The mother becomes immunized during pregnancy and produces Rh antibodies which pass back through the placenta and attack the foetus, producing the condition known as haemolytic disease of the newborn. From the point of view of immunization it is interesting to note that during their

occur more frequently in pathological sera and in sera from cases of rheumatoid arthritis (Waller and Vaughan 1956). The substances responsible for these reactions are thermo-stable and are not, apparently, related to complement.

Sera possessing these properties can cause agglutination of sensitized red cells which would not otherwise occur. They constitute another property of serum which is a reactant in some antigen-antibody reactions. In the future it seems probable that more use will be made of these properties of serum.

Immunity and Immunization

There are two kinds of immunity:

1. NATURAL IMMUNITY. This is innate reaction to disease. Natural immunity varies from species to species of animal; some animals are susceptible to diseases to which Man is very resistant, and vice versa.

2. ACQUIRED IMMUNITY. Acquired immunity is of two kinds, (*a*) passive, (*b*) active.

(*a*) *Passive immunity*. It is not proposed here to consider passive immunity in detail. It is a type of immunity which results from administration to the animal concerned of serum or serum products from an immune animal. Passive immunity may arise from the parenteral administration of the serum from the immune animal or naturally by its passage through the placenta or sometimes via the breast milk, or colostrum. Blood group antibodies can cross the placental barrier and appear in the foetal blood stream. Anti-A and anti-B frequently cross from the maternal to the foetal serum, as do other antibodies, which may result in disease in the child. Breast milk may also contain blood group antibodies which it is thought may affect the child.

(*b*) *Actively acquired immunity*. This results from the action of various factors which tend to increase resistance of the animal to disease. These may result from alterations in the tissues or mobilization of cellular elements, or other factors of non-specific kind, or they may result in the production of antibodies secreted into the serum and present in the tissue fluids as a result of the natural or artificial administration of antigens to the animal.

One of the characteristic features of immunization of an animal with an antigen is the production of an antibody. The first dose of antigen given to the animal may produce a minimal response, that is, the antibodies may not readily be detectable, but a subsequent dose of antigen results in a rise in antibody titre, far greater than the first injection produced. This is related also to the time interval between the first and second doses, and any subsequent doses. If one dose only is given the antibody produced may gradually fade from the animal's

newborn, the blood of mothers and fathers, or mothers and babies, shows a higher degree of ABO compatibility than in the normal population. Many series have been published; Stratton (1953) found 67 per cent of compatible matings in normal families compared with 82 per cent where the mothers were Rh negative and had Rh antibodies. It may be that in incompatible matings the foetal erythrocytes are destroyed so rapidly that the chance of maternal immunization is greatly reduced.

References

Aminoff, D., Morgan, W. T. J., and Watkins, W. M. (1950). Studies in Immunochemistry. 8. The isolation and properties of the human blood group A substance. *Biochem. J.*, 46, 426.

Bessis, M. (1955). Phase contrast microscopy and electron microscopy applied to the blood cells. *Blood*, 10, 272.

Billingham, R. E., Brent, L., and Medawar, P. B. (1956). Quantitative studies in tissue transplantation immunity. III. Actively acquired tolerance. *Philosophical Transactions of the Royal Society of London*, Series B, 239, 357.

Booth, P. B., Plaut, Gertrude, James, J. D., Ikin, Elizabeth W., Moores, Phyllis, Sanger, Ruth, and Race, R. R. (1957). Blood chimerism in a pair of twins. *B.M.J.*, i, 1456.

Bordet, J., and Streng, O. (1909). Les phenomènes d'absorption et la conglutinine du sérum de boeuf. *Zbl. Bakt.*, 49, 260.

Boursnell, J. C., Coombs, R. R. A., and Rizk, Vivien (1953). Studies with marked antisera. Quantitative studies with antisera marked with iodine[131] isotope and their corresponding red cell antigens. *J. Biochem.*, 55, 745.

Boyd, W. C. (1939). Blood groups. *Tab. Biologicae*, 17, 113.

Boyd, W. C. (1956). *Fundamentals of Immunology*. 3rd Ed. Interscience Publishers.

Boyd, W. C., and Bernard, H. (1937). Quantitative changes in antibodies and globulin fractions in sera of rabbits injected with several antigens. *J. Immunol.*, 33, 111.

Coombs, A. M., and Coombs, R. R. A. (1953). The conglutinin phenomenon. IX. Production of immune O conglutinin in rabbits. *J. Hygiene*, 51, 509.

Dakin, H. D., and Dale, H. H. (1919). Chemical structure and antigenic specificity. A comparison of the crystalline egg albumins of the hen and the duck. *Biochem. J.*, 13, 248.

Diamond, L. K. (1944). Progress Report of Committee on Medical Research of the Office of Scientific Research and Development. O.E.M. cmr. 384.

Dunsford, I., Bowley, C. C., Hutchinson, Ann M., Thompson, Joan S., Sanger, Ruth, and Race, R. R. (1953). A human blood group chimera. *B.M.J.*, ii, 81.

Filitti-Wurmser, Sabine, Aubel-Lesure, G., and Wurmser, R. (1953). Constantes de sédimentation des iso-agglutines β (A_1O), β (OO) and β (A_1A_1). *J. Chim. Phys.*, 50, 236.

first pregnancy Rh negative mothers do not normally produce Rh antibodies, even though the first child is Rh positive so that first children are rarely affected with the disease. They more commonly produce them during the second and subsequent pregnancies. Where Rh antibodies are produced during the first pregnancy it is almost always found that there has been an initial antigenic stimulus, usually an Rh positive blood transfusion, at an earlier period of the mother's life.

CASE 1

> Mrs. V was Rh negative and had potent Rh antibodies in her serum. Her husband was Rh positive, homozygous R_1R_1, and her baby when born was found to be Rh positive, and even though it was the first child it was severely affected with haemolytic disease and subsequently died. It was found that ten years previously the mother had received an Rh positive blood transfusion. This acted as the first stimulus and the first child consequently produced a very potent response in the mother.

Another interesting feature of the condition is the frequency with which it occurs. Calculations from the occurrence of the condition in Manchester showed that 1 in 17 matings of Rh negative mother × Rh positive father produced children affected with haemolytic disease of the newborn. It is thus obvious that women vary in their susceptibility to antigenic stimuli. Some readily produce antibodies and, indeed, occasional cases do occur, even in the absence of previous blood transfusion, in which mothers are immunized by a first pregnancy. Other mothers never become immunized, even after a large number of pregnancies, and some become immunized after four, five, six or more pregnancies of Rh positive children. This is a common feature of the production of antibodies generally. Another feature of haemolytic disease which is of interest from an immunological point of view is the occurrence of an anamnestic response. That is to say, in a case where a mother is Rh negative and has Rh antibodies in her serum, resulting from a previous pregnancy, there might be an increase in titre of the mother's Rh antibodies during pregnancy, even though the foetus is Rh negative.

It is not only Rh negative mothers who have children affected with haemolytic disease of the newborn. Mothers who are Rh positive may be immunized to some antigen not present on their own red cells. It appears to depend upon the susceptibility of the mother to immunization whether or not she produces such an antibody.

Heterospecific pregnancy, a term introduced by Hirszfeld, is one where the red cells of the foetus would be agglutinated by the anti-A or anti-B of the maternal serum. Thus a mother of group O would have an incompatible or heterospecific pregnancy if the child were group A or group B, or compatible if the child were group O. In families in which a child is affected with haemolytic disease of the

Filitti-Wurmser, Sabine, Jacquot-Armand, Y., Aubel-Lesure, G., and Wurmser, R. (1954). *Physico-chemical studies of human isohaemagglutination. Ann. Eugen.*, **18**, 183.

Grove-Rasmussen, M., Shaw, R. S., and Dreisler, Nina (1955). Stimulation of Rh antibodies in an Rh negative patient with hæmophilia. *New Eng. J. of Med.*, **252**, 673.

Grubb, R. (1956). Agglutination of erythrocytes coated with "incomplete" anti-Rh by certain rheumatoid arthritic sera and some other sera. The existence of human serum groups. *Acta Path. Microbiol. Scand.*, 39, 195.

Kabat, E. A., and Mayer, M. M. (1948). *Experimental Immuno-chemistry.* Illinois: Charles C. Thomas.

Kabat, E. A., Baer, H., Day, R. L., and Kraut, V. (1950). Immunological studies of blood groups. XI. Species specific differences among blood group A substances. *J. Exp. Med.*, **91**, 433.

Landsteiner, K. (1945). *The Specificity of Serological Reactions.* Cambridge: Harvard University Press.

Lepow, I. H., Ratnoff, O. D., and Pillemer, L. (1956). Elution of an esterase from antigen-antibody aggregates treated with human complement. *Proc. Soc. Exp. Biol. Med.*, **92**, 111.

Loghem, J. J. van, Bartels, H. L. J. M., and Hart, Mia van der. (1949). La production d'un anticorps anti-C^{w} par immunisation d'un donneur bénévole. *Rev. d'Hem.*, **4**, 174.

Mayer, M. M., and Levine, L. (1954). Kinetic studies on immune hemolysis III. Description of a terminal process which follows the Ca^{++} and Mg^{++} reaction steps in the action of complement on sensitized erythrocytes. *J. Immunol.*, **72**, 511.

Milgrom, F., Dubinski, S., and Wozniczko, G. (1956). Human sera with "anti-antibody". *Vox Sang.*, **1**, 172.

Murray, J., and Clark, E. C. (1952). Production of anti-Rh in guinea pigs from human erythrocyte extracts. *Nature*, 169, 886.

Nicholas, J. W., Jenkins, W. J., and Marsh, W. L. (1957). Human blood chimeras. *B M.J.*, i, 1458.

Pillemer, L., Blum, L., Lepow, I. H., Ross, O. A., Todd, E. W., and Wardlaw, A. C. (1954). The properdin system and immunity. I. Demonstration and isolation of a new serum protein, and its role in immune phenomena. *Science*, **120**, 279.

Renton, P. H., and Hancock, Jeanne, A. (1957). Antibody-like effects of colloidal silica. *Vox Sang.*, **2**, 117.

Stratton, F. (1953). *Clinical Genetics.* Ed. A. Sorsby. London: Butterworth and Co. Ltd.

Streng, O. (1930). Immun O Konglutinin-Anti-Komplement A. *Acta Path. Microbiol. Scand.*, **20**, 411.

Waller, M. V., and Vaughan, J. H. (1956). Use of anti-Rh sera for demonstrating agglutination activation factor in rheumatoid arthritis. *Proc. Soc. Exp. Biol. Med.*, **92**, 198.

Wiener, A. S. (1951). Origin of naturally occurring haemagglutinins and haemolysis. A review. *J. Immunol.*, **66**, 287.

Witebsky, E., Klendshoj, N. C., and McNeil, C. (1944). Potent typing sera produced by treatment of donors with isolated blood group specific substances. *Proc. Soc. Exp. Biol. Med.*, **55**, 167.

CHAPTER II

AGGLUTINATION TESTS

In blood grouping work the observable results by which it is known that antigen-antibody reactions have occurred are either erythrocyte agglutination or haemolysis. The purpose of this chapter is to describe how agglutination tests should be carried out.

Materials

Measurements in blood grouping work are undertaken using marked Pasteur pipettes. A drawing of such a pipette is shown in Fig. 1. It is usually about 4 inches long with a narrowing tip about 2 inches long and is fitted with a small rubber teat. A stock of these pipettes can be kept ready for use in a sterile container. The volume of serum or cell suspension used in agglutination or other tests varies to some extent from test to test, but many tests require about 0·04 ml. and it is often valuable to have a Pasteur pipette graduated for this volume. This is done by making a circular pencil mark on the narrow end of the pipette about 1 inch from the tip. 1 ml. of saline is then put into a tube and tests are made to determine whether 25 volumes can be removed from it using the pipette; the mark is adjusted accordingly. This is not an accurate method of dispensing volumes of serum or cells, but it is sufficiently accurate for most blood grouping work. Drops of liquid or cells should never be used. They vary too much in volume and it is almost as speedy to place a measured volume in the tube as it is to put a drop in each tube.

If still greater accuracy is required, graduated 1 ml. pipettes are used. In some work the use of sterile graduated pipettes is essential; for example, in the titration of complement where accurate quantitative work is needed. If accurate titrations of blood group antibodies are required, it is necessary to make up the dilutions using graduated pipettes. In making up dilutions of antisera it is always advisable to use graduated pipettes; similarly, graduated pipettes should be used for making dilute suspensions of red cells from packed erythrocytes. Using a Pasteur pipette, a 3 or 5 per cent strength of suspension can be judged fairly accurately, with experience, by its colour and density when viewed in the pipette.

Disposable pipettes are very useful and are shown in Fig. 1. The first one employs a disposable sterile capillary which is placed in the end of a tube with a teat on it. Very small quantities of fluid can be

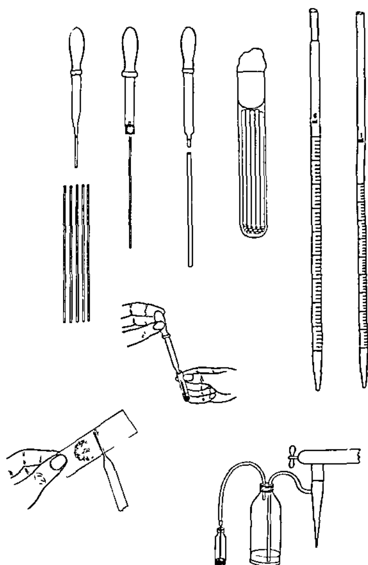


FIG. 1. Pipettes—Suction Pump, etc.

blood collection, as in the others, care should be taken to use different sterile apparatus for each person to avoid the risk of homologous serum jaundice or syringe jaundice. Syringes, etc., should be dry sterilized or rinsed in saline to avoid haemolysis.

Blood from infants may be obtained by intravenous puncture, or heel stab. Cord blood should be collected from the umbilical vein using a syringe and needle; the cord should not be "milked" to obtain a sample. Avoid Wharton's jelly.

Blood when collected may be run into a neutral isotonic fluid, be allowed to clot, be defibrinated, or be mixed with an anticoagulant.

It is not generally advisable to run blood into a neutral isotonic fluid, since the subsequent red cell suspensions are very liable to contain small clots.

Clotted blood may spontaneously separate from the sides of the container, but the tube or bottle may need ringing. That is, using a sterile applicator stick or glass rod, separate the clot from the sides of the tube or bottle so that the clot will retract completely. Defibrinated blood is prepared by whisking the blood with a hooked glass rod immediately it has been collected. The rod is held in the finger and thumb and twirled in the blood as it clots. When clotting is complete a mass of fibrin is removed.

Various anticoagulants have been suggested for the collection of blood. Firstly, A.C.D. solution. One volume of this is mixed with 4 volumes of whole blood; 25 ml. containers should be kept ready prepared with 4 or 5 ml. of A.C.D. mixture already in them so that the appropriate quantity of blood can be added from a syringe. Even where drops of blood are put in A.C.D. mixture these proportions should be adhered to. 3·8 per cent trisodium citrate is a very useful medium into which to collect small quantities of blood, or drops of blood. Heparin, or a heparin-saline mixture, has also been used.

It is sometimes considered that it is an advantage to add antibiotics to the [illegible] may be added (Callan, 1955).

The relative merits of clotted and defibrinated blood and that collected in anticoagulant, depend upon the survival and preservation of the red cells themselves, as well as on the preservation of the antigens. With clotted blood samples sufficient free cells can be obtained to make a good erythrocyte suspension. Large quantities of cells are not available and if these are required blood should be collected as defibrinated blood. In both these samples serum is available. The red cells so obtained have not been contaminated by any extraneous substances such as anticoagulant. Red cells collected in this way, however, are not well preserved at 4° C.; much better preservation is obtained if A.C.D. is used, which is the best preservative for this

wool plug and sterilized. A small glass tube with a ground glass end (a part of a blood giving set), and teat, is pushed into the end of these and enables them to be withdrawn aseptically from the tube. These form a very useful means of withdrawing serum, saline, cells, etc., aseptically from containers.

Microscope slides used are of the 3×1 inch standard type. Opal glass tiles can be obtained of varying thicknesses, and if it is desired to use them for titration purposes 12×3 inch tiles are a convenient size.

Tubes of various sizes are employed, the commonest being a precipitin tube $2 \times \frac{1}{4}$ inch or $2 \times \frac{3}{8}$ inch. For the antiglobulin tests a $3 \times \frac{1}{2}$ inch tube is used. Plastic tubes of various sizes can be obtained and are useful in that they never smash in the centrifuge, and if made of nylon can be autoclaved and kept sterile.

Bottles of various sizes are also useful and screw-cap bottles of 1, 2, 5, 20, 50 and 100 ml. should be kept as sterile containers in the laboratory. These are prepared with a few drops of saline in the bottom and sterilized with the caps screwed tightly down. Plastic bottles are also valuable and occasionally these can be obtained in materials which may be sterilized, but generally this is not so and polythene bottles have to be employed; sterilization here presents a problem.

Disposable applicator sticks are very useful for ringing clots and for mixing the cell-serum samples on a slide.

Sterile isotonic saline solutions are prepared by autoclaving in glass bottles and may have rather a low pH although no buffering power. If buffered saline is desired, veronal buffered saline, as recommended by Pillemer *et al.* (1956), may be employed (p. 51).

Sterile anticoagulant solutions, A.C.D., or trisodium citrate, are made up in 20 ml. containers. Once a bottle is opened and used it is discarded, since the solution readily becomes infected.

A.C.D. Solution

Disodium hydrogen citrate	2·50 gm.
Glucose	2·00 gm.
Distilled water	100 ml.

Trisodium Citrate Solution (isotonic)

Trisodium citrate, $2Na_3C_6H_5O_7,11H_2O$	3·8 gm.
Distilled water	100 ml.

Erythrocyte Suspensions

Blood may be obtained from individuals in various quantities, by pricking the finger or ear, or by intravenous puncture with a syringe (up to 50 ml.), or with a taking set and bottle (up to 500 ml.). When collecting blood, all the containers and apparatus should be sterile and the blood should be removed aseptically. This is not possible, generally speaking, with finger or ear lobe pricks. Particularly in this form of

solutions are autoclaved, or in which the red cells are stored. It is only effective on washed red cells. Freezing itself has an effect on colloidal silica which tends to increase this effect. When storing glycerol treated red cells at −25° C., therefore, it is desirable that polythene or plastic bottles should be used. Citrate and other solutions used for preserving the red cells and for restoring them to saline suspension should be prepared by autoclaving in plastic bottles, or sterilized by Seitz filtration. No solutions or substances used in this work should be autoclaved or frozen in glass bottles.

Red cell suspensions made from blood collected as above are normally suspended in saline. In the case of clotted blood sufficient loose cells are available to make the suspension, but in the case of anaemic persons this may not always be so and the clot may have to be broken up in order to obtain sufficient free cells. Rosenfield (1957) [illegible]
[illegible]
[illegible]

Cells should be washed twice in twenty times their volume of normal saline, which will ensure that not more than 0·5 per cent of serum is present in the cell pack. This supernatant saline should also be free from haemoglobin; if it is not, further washing should be made until it is free. The fact that haemoglobin was present in the washings indicates that the cells may be old or they may be unsuitable for use. The final suspension is prepared from the cell pack using graduated pipettes or Pasteur pipettes in a concentration from 3 to 10 per cent. according to the test in which it is to be used. The bulk cell suspension may now be left for a few minutes on the bench in order to permit any small clots to sink to the bottom of the tube and the supernatant suspension decanted off.

Antisera

When blood is collected from Man or animals for the purpose of obtaining the serum it is usual to allow blood to clot, although it may be defibrinated. If this is a small quantity of blood, say 20 ml., the bottles may be spun in the centrifuge. Where larger quantities of blood (200–250 ml.) have been collected, these may be centrifuged and the serum collected, or the bottle may be hung up in the cold-room with a standard giving set inserted into it and the serum run off for twenty-four hours into a container below. Both these methods give about the same yield of serum, but a greater yield of serum still can be obtained if silicone coated bottles are used. All these procedures should be carried out with strict aseptic [illegible]

[illegible] C. but when in daily [illegible] +4° C. for as short a period as possible. The lower temperature of storage (−25° C.)

purpose, but has not such good anticoagulant properties as tri-sodium citrate. Deterioration of erythrocyte antigens is referred to in Chapter V.

Small quantities of blood in the form of drops of blood should not be run into large quantities of anticoagulant because the pH of the red cells may be altered thereby. Heparin is not satisfactory since cells do not preserve well in heparin and it is also violently anticomplementary, and even washed red cells may retain some of this activity.

Blood samples for anthropological blood group determination may present special difficulties. In the first place it may be necessary to collect them by finger prick methods; there may be difficulty in preserving sterility and tropical conditions may appertain at the site of collection. It would seem that the best solution to these problems is to use an A.C.D. mixture containing antibiotics and to fly samples back to the laboratory as soon as possible. Many workers have their own particular solutions which they consider to be superior to others.

If red cell samples have to be preserved for long periods of time they should be kept frozen solid at −25° C. Mollison (1956) recommends the following method:

Blood is collected into A.C.D. mixture. This is then centrifuged and the plasma supernatant discarded and replaced by an equal volume of glycerol solution (40-vols. glycerol to 60 vols. 5 per cent trisodium citrate). The mixture is then partitioned into a number of small tubes and these are placed at −25° C. When the red cells are to be used, a tube is thawed in warm water at 37° C. and the red cells freed from glycerol by washing in successively more dilute solutions of glycerol. A stock of suitable solutions is suggested as being 16, 8, 4 and 2 per cent glycerol (W/V) in 3 per cent citrate. The cells should then be washed twice in saline and are ready for use.

Crawford, Cutbush and Mollison (1954) consider that cells stored in this way for one year react almost as well as fresh cells, and this appears to apply to the reactions of all blood group antigens. It has been our experience that red cells stored in this way react almost, but not quite, as well as fresh cells, certainly after a period of six months' storage and probably in most cases for twelve months'. Certain receptors are less well preserved than others, e.g. autoagglutinin receptors, H and O.

Certain difficulties arise from time to time when frozen cells are restored to the saline medium by the successive washes in decreasing concentrations of glycerol solution. Sometimes it is found that the red cells on being brought up into saline spontaneously agglutinate to a variable extent when suspended in the final saline solution. Stratton

TABLE 4. ANTI-S SERUM RECONSTITUTED FROM FREEZE-DRIED PRODUCT USING DISTILLED WATER AND ACIDIFIED WATER

Method of reconstitution	*pH of serum*	*Test cells*	*Titre at 16° C.* 1	2	4	8	*Control*
Add Distilled water ..	9·2	S+	++++	+	−	−	−
		S−	+++	−	−	−	−
Add N/50 HCl at 0° C. ..	7·2	S+	+++++	++	−	−	−
		S−	−	−	−	−	−

absorption they will need to be sterilized before they can be used. Heavy contamination will prevent the serum being used as a blood grouping serum and panagglutinins may be present. If they have retained their specificity and contamination is only slight, sterilization may be carried out by Seitz filtration. It is desirable to avoid the need for Seitz filtration of sera if at all possible because with many sera there is a loss of titre of about one or two tubes following Seitz filtration. Antiseptics may be added to sera—0·1 per cent sodium azide—to prevent bacterial growth due to accidental contamination during their use. If, however, the sera are kept in an ice tray for bench work, or in the refrigerator at 4° C. for the minimum possible time and largely preserved frozen solid, accidental contamination will not result in deterioration of the antibody potency of the serum or in non-specific activity.

Anti-A and anti-B active as haemolysins are inactivated before use as blood grouping sera. Anti-Le^a, anti-Le^b and anti-Jk^a may depend on complement for optimum activity and should not normally be inactivated. *Inactivation* may make fresh serum anticomplementary, as may storage (p. 54).

Absorption of antisera may be necessary to remove anti-A, anti-B or other unwanted antibodies from the serum or, in the case of animal sera, to remove species specific agglutinins. A typical example of the absorption of animal sera is the preparation of anti-M and anti-N. The most frequent reason for which human sera are absorbed is to remove anti-A and anti-B from the serum in order to leave the specific atypical antibody active against any ABO group of red cells.

TECHNIQUE OF ABSORPTION FOR THE REMOVAL OF ANTI-A AND ANTI-B OR OTHER ANTIBODIES

1. Select suitable cells for the absorption; blood collected into A.C.D. is suitable. If anti-A is to be removed the absorbing cells must be sub-

prevents bacterial contaminants from multiplying and minimizes loss of antibody potency. If the refrigerator in which the sera are frozen solid *fails to such an extent that the sera inside thaw out, they should all be* removed and the bottles gently shaken up before the sera are refrozen. This is because during thawing of the frozen sera, layering takes place and the concentrated protein sinks to the bottom of the container. If such a layered mixture is refrozen the bottom layer forms a sticky mass in which denaturation may occur. Sera when stored at −25° C. will lose their complementary activity gradually over a period of weeks or months and may later develop anticomplementary properties. Some sera are not readily preserved by storage frozen at −25° C., for example, anti-Le^a and anti-Jk^a. Preservation at −70° C. may be advantageous (p. 299).

Freeze-drying is another method by which blood group antibodies can be preserved. Anti-Rh sera preserved by this means have been stored without serious loss of potency for more than five years in our laboratory. In an experiment, a sample of anti-Rh agglutinin so dried was placed in a bath of boiling water for a period of one hour and then reconstituted and tested; it was found that there was no loss of antibody potency.

During the drying process CO_2 is removed from the serum and consequently when reconstituted with distilled water the serum has a much higher pH than it had originally. The pH of reconstituted dried serum may be more than pH 9. It is sometimes an advantage, therefore, to reconstitute using N/50 HCl at 0° C. so that the final mixture is about pH 7. Some examples of anti-Le^a and anti-Jk^a are not at all well preserved by this means, even when reconstituted at 0° C. with acidified water. Preservation of complement may be affected by freeze-drying and reconstitution with water (Table 3).

TABLE 3. EFFECT OF FREEZE DRYING ON WHOLE C′ TITRE OF SERUM

Method of reconstitution	*pH*	*Complement titre 50% units/ml.*
Distilled water	9·5	70
Carbonic acid water	7·0	90
N/50 HCl at 0° C.	7·4	90
Control fresh serum	8·2	90

Some antisera may give different results if reconstituted with distilled water instead of acidified water. Table 4 illustrates this and shows loss of potency and specificity at high pH.

If blood grouping sera become contaminated during collection or

9. Repeat the absorption if necessary. Half a volume of packed cells will usually be found sufficient for this.

ALTERNATIVE TECHNIQUE FOR ABSORPTION. 1, 2, 3 4 and 5—as above.

6. Mix equal volumes of cells and serum together at room temperature. The cells and serum should be mixed together as quickly as possible and the container is then turned upside down two or three times, following which it is immediately centrifuged and the serum removed.

7. Test the serum again A and/or B cells of appropriate other groups by the slide agglutination technique (as used in ABO grouping, p. 116).

8. If the agglutination seen in (7) is stronger than ++, repeat (6) and retest. The process is repeated until such time as only a ++ agglutination is seen on a slide test.

9. Absorb the serum with half a volume of packed washed cells for 30 minutes at 4° C.

10. Centrifuge and retrieve the serum.

11. Absorb with half a volume of packed washed cells at 37° C. for half an hour.

12. Centrifuge and retrieve the serum.

13. Test the absorption as in (8) of the preceding method.

The purpose of the rapid absorption at the beginning is to try to prevent antigen-antibody [illegible] of the red cell in [illegible] upturned very [illegible] but not too rapidly. This is done because it is thought that the return of unwanted antibodies to animal or human sera may be due to break-up of avulsed antigen-antibody complex in the serum. It is also a useful method when absorbing animal sera, such as anti-M and anti-N, where species specific and other unwanted components may require repeated absorption for their removal.

Absorption at 4° C. as well as at 37° C. is recommended, since if absorption at 37° C. alone is carried out, especially in the case of removing anti-A antibody from serum, anti-A activity may be removed but an anti-A_1 antibody active only at 16° C. may remain behind. Tests should also be made in serum media to make sure that all incomplete anti-A_1 has been removed from the serum; similarly, if A_2 cells were used for absorption difficulties might occur. Special methods are required if absorption is to be undertaken with minimal reduction of complement titre (p. 54).

When sera are [illegible] of [illegible] to [illegible] per volume, so that if a serum is absorbed with

group A_1; A_2 cells are unsuitable. If the serum to be absorbed is group O, it is probably best to absorb with a mixture of washed A_1 and B cells, although alternatives are to use A_1B cells, or to absorb with A_1 and B cells separately in two successive absorptions. The cells must, of course, be of such other groups as not to react with the antibody which it is desired to leave behind in the absorbed serum and, in the case of anti-D, rr cells should be used.

2. Confirm the correctness of the ABO and other appropriate groups of the absorbing cells by suitable tests.

3. Wash the cells three times in a large volume of saline and pack them. If a mixture of A_1 and B cells is to be used they must be washed before mixing.

4. Inactivate the serum by heating at 56° C. for half an hour in order to destroy complement which might otherwise lead to haemolysis during the absorption. If the antibody present in the serum is one which requires complement for its action, this step will have to be omitted (e.g. certain anti-Lewis sera) and the rapid absorption method used.

5. Cool the serum to 37° C. or below; if it is hot it may haemolyse the absorbing cells.

6. Add one volume of serum to one volume of packed cells; mix well together and incubate for a period of half an hour at 37° C. followed by a further period of one hour at 4°–6° C. If a mixture of A_1 and B cells is used two volumes of the mixture should be used to one volume of serum. If the volume of serum being absorbed is large, the period of incubation at 4°–6° C. should be increased to allow for the slow cooling of the mixture to this temperature.

7. Centrifuge and harvest the absorbed serum. The centrifugation should be carried out at high speed so that the red cell ghosts and other debris are removed from the serum.

8. Test the serum with A_1 and/or B cells of other groups not reacting with the antibody concerned to see whether the absorption has been sufficient. This test should be carried out by two techniques; firstly by the tube technique at 16° C., since this will give optimal results with anti-A and anti-B, and, secondly, by the technique by which it is intended to use the absorbed serum, since although the serum may be intended for use at 37° C. it is not always possible to avoid some cooling of the tubes when the tests are read. Serum containing anti-A or anti-B which was still active at 16° C., although giving no reaction at 37° C., would be unsafe to use. If the absorption was done with the object of removing some antibody other than anti-A and anti-B, the absorbed serum must, of course, be tested against cells with the corresponding antigen and this should be done by whatever technique will best detect the particular antibody concerned. Tests by the technique by which it is intended to use the absorbed serum must also be included.

Elution is used:

1. In cases of acquired haemolytic anaemia to identify antibodies absorbed on to the patient's erythrocytes.
2. Similarly, to identify antibodies absorbed on to cord cells in cases of haemolytic disease of the newborn (Cases 33 and 68, pp. 196 and 262).
3. In the isolation and identification of antibodies where several are present in the same serum and to help to characterize new antibodies.
4. In the detection of rare subgroups of A and B. This may be the only method by which the presence of A or B group antigens may be detected on the red cells; we have found it most useful in Case 27 (p. 111).
5. As a research procedure in the investigation of antigen-antibody reactions (p. 8).

Physical Conditions affecting Antigen-antibody Reactions

a. Method of bringing antigen and antibody into contact.
b. Ionic strength of the medium.
c. pH of the medium.
d. Duration of the test.
e. Volumes of reagents.
f. Temperature of the test.
g. Strength of the red cell suspension.
h. Presence of additional substances during performance of the test.

When red cells containing the appropriate antigen are mixed with antisera containing homologous antibody, agglutination may occur. The nature of the antibody is obviously concerned in this, for unless special measures are taken incomplete antibodies will not cause agglutination of red cells, but when albuminous, or other, media are used in which to suspend the cells, or when the anti-globulin test is used, erythrocyte agglutination can be obtained. These factors can influence agglutination, and if the physical conditions of the test are not right, although antigen and antibody are present in optimal proportions, nevertheless the expected result will not occur.

a Method of bringing antigen and antibody into contact. Of all the factors concerned in obtaining satisfactory erythrocyte agglutination when red cells are mixed with antiserum, the particular method by which this is done is important. Although an antigen-antibody reaction will give a positive result by more than one particular method, nevertheless it will often be found that one particular one does give results which are superior to others and this will be the one which is generally employed for that particular antigen-antibody reaction.

ten times its own volume of packed red cells it should be considered to have been diluted 1 in 2 by this procedure alone.

Anti-A and anti-B can also be neutralized in serum by the addition of A and B substances. This is not generally recommended because of the difficulty of neutralizing incomplete anti-A or anti-B. Sera in which atypical antibodies, such as anti-Rh, occur, often contain high titre immune anti-A and anti-B antibodies· It is, however, a useful method where suitable group A or B cells are not available for absorption of the serum. If serum from a group O person containing anti-*k* is to be absorbed, for example, cells of group A,KK, or B,KK, may not be available and the anti-A or anti-B may have to be neutralized using specific substances. Anti-e is another case where this may be necessary (p. 183).

ELUTION OF ANTIBODIES

This is a process for the preparation of purified solutions of antibody in saline. The method recommended is essentially that of Landsteiner and Miller (1925). One volume of a 20 per cent suspension of red cells is added to 10 or 15 volumes of serum containing the antibody in question. The mixture is then incubated for one hour at the optimum temperature. The cells are spun down, the serum removed and the cells washed three times in normal saline. A small quantity of saline is then added to the packed cells, equivalent to about the volume of the packed cells themselves.

This is then heated at 58° C. for five or six minutes, being constantly shaken. A thermometer should be placed inside the tube to ensure that the sensitized cell saline mixtures do actually attain the correct temperature. At the conclusion of the heating the tube is placed in a centrifuge cup filled with water at 58° C., centrifuged at high speed and the supernate removed. This should be done as rapidly as possible, since if the eluted antibody is in contact with the cells at temperatures below 58° C. it may start to recombine with the antigen. Rapid centrifugation also helps to throw down red cell stroma which could neutralize the activity of the eluate.

This supernate will contain free haemoglobin and the temperature and time have been increased beyond those recommended by Landsteiner and Miller with the object of liberating some haemoglobin from the red cells because this stabilizes the eluate. Many other techniques for making eluates have been described, but certainly that of Landsteiner and Miller has stood the test of time. It is possible to concentrate an eluate by freezing it and allowing it to thaw out slowly, when it becomes more concentrated in the lower portion of the solution; the upper part is, therefore, carefully pipetted off and the lower part is used for further tests; the division is seen when the tube is held to the light.

required for sensitization may be different from those required for agglutination.

Capillaries have been recommended and are used for a number of blood grouping tests (Chown and Lewis, 1946). Care has to be taken when using capillaries to avoid false positive results. We have found, however, that red cells treated with papain by the method described on p. 40 do give a good agglutination with suitably diluted Rh antisera at 37° C. when allowed to run down capillary slopes at an angle of 60°, but anti-A and anti-B give only weak results with papainized cells using this technique.

b. Ionic strength of the medium. We have not found any advantage in using other than isotonic saline in blood grouping work.

c. The pH of the medium. The pH of the test is subject to a fairly wide range of variation without materially affecting the final result of the red cell agglutination. The range of pH 7 to pH 8·5 is satisfactory in the vast majority of cases. Within this range complement is active, but below and above this range the activity of complement is reduced (p. 53). Furthermore, raising or lowering of the pH much beyond these levels might affect the agglutination reactions. Experience leads us to believe that unusual results might occur outside the range of pH 7 to pH 8·5—sometimes advantageous ones, for Gardner (1949) has suggested that acidification of the serum might assist in the detection of antibodies in cases of acquired haemolytic anaemia (cf. Table 4).

d. The duration of the test. In an agglutination test, two periods of time must be considered; firstly, the time necessary for the antigen-antibody reaction to take place, and secondly, the time necessary for the sensitized cells to sediment to the bottom of the tube and agglutinate. In sensitizing cells for the Coombs test, only the first factor need be considered. Antibodies vary widely in the length of time needed for their reaction to go to completion, but for practical purposes it would be fair to say that in most cases the reaction is complete within 40 mins., though we have very rarely encountered antibodies which required two hours to sensitize cells in the Coombs test. The period needed for sedimentation in the agglutination test varies with the volume, temperature and suspending medium. In saline at 37° C. an hour and a half is satisfactory; at room temperature, a little longer; in albumin, or serum-albumin suspension [illegible] ° C. [illegible] l. of [illegible]. In [illegible] Our [illegible] half [illegible] of either test is reduced below this some loss of

Opal tiles, microscope slides, tubes of various sizes and capillaries have all been used on or in which to mix red cells and antisera.

Tiles and slides have to be confined to those antigen-antibody reactions known to occur by this method and where maximum agglutination might be expected to occur fairly briskly; that is, within 6 minutes of the cells and serum being mixed on the slide at room temperature. For example, with anti-P and P-positive cells, although agglutination will occur on a slide it is slow to develop. Even certain weaker anti-A and and anti-B agglutinins may not reach maximum activity with homologous cells within this time. The method cannot be used at 37° C. because of the drying up of the reagents. Various means have been described by which this may be overcome, e.g. by the use of moist chambers, but they find no place in routine work. If the conditions of *the test are modified certain blood group reactions are possible which* would not otherwise occur—if enzyme treated cells are used, or if an albumin instead of a saline suspension is employed. If a sandwich technique is used weak Rh antibodies will, in the presence of albumin, agglutinate Rh-positive red cells, whereas a positive result might not occur on an open slide. The only difference here is the different condition of the test; in the former the cells are in close juxtaposition and their movement is reduced.

The disadvantage of tiles is that the test cannot be examined under the microscope. Many people are prepared to read the results on a tile with a hand lens; we feel that results are best examined microscopically wherever possible. Weak positive results may be indistinguishable from negative ones to the naked eye and many false positive results, e.g. clots and comets, have typical microscopical appearances. Consequently, tiles are not of great value in blood grouping work.

Tubes are generally used for blood grouping work. They can be used for large-scale work, they permit the minimum of evaporation of reagents, they are readily used at any temperature in a water bath or incubator, and the cells may be allowed to sediment by gravity for varying periods of time, or the tubes may be centrifuged. The cell deposit can be inspected macroscopically by tapping the tube, or microscopic reading can be done. An albumin replacement method has been developed by the Sheffield workers (Dunsford and Bowley, 1955) in which after incubation of the serum with a saline cell suspension the saline is replaced by albumin solution. We have used a layering technique to detect the presence of saline agglutinins in anti-Kell sera. The conditions of this test are similar to those in the sandwich, i e. the cells are in close juxtaposition and their movement is restricted. It seems that under these conditions, with certain antibodies, linkage of the cells on to one another to produce agglutination is more likely to occur. If the albumin test is carried out in tubes the conditions

g. The strength of the cell suspension. This affects the strength of agglutination observed. If the dose of cells is too great the antibody will be absorbed on to a large number of cells in small quantity on each, so that the agglutination may be quite feeble, or even indiscernible. If a thinner suspension is used then the antibody is absorbed in greater quantity on to each cell and the result is much better. It is necessary to strike a balance between too few cells which make the technique difficult, and too many cells (Table 7). Generally 3-5 per cent suspensions are used.

TABLE 7. EFFECT OF THICKNESS OF CELL SUSPENSION ON TITRE OF RH ANTIBODY

Thickness of suspension	*Titre*									*Score*
	2	4	8	16	32	64	128	256	*Control*	
%										
2	+++++	++++	+++	++	++	+	−	−	−	17
5	+++++	++++	+++	++	+	w	−	−	−	15
10	++++	+++	++	+	−	−	−	−	−	10
20	+++	++	+	+	−	−	−	−	−	7

h. The presence of additional substances during the performance of the test. These may be necessary for the antigen-antibody reaction to occur, or more commonly, for it to be detected; e.g. complement in the case of cold incomplete anti-H and albumin for the final agglutination reaction in the case of certain incomplete antibodies.

Saline and Serum-albumin Agglutination Tests

The saline and serum-albumin agglutination tests are similar, but differ in that in the saline agglutination test the red cells are suspended in isotonic saline and the antiserum is diluted in this fluid. In the serum-albumin test the red cells are suspended in the serum albumin mixture though if the antiserum is diluted it is diluted in serum. Whereas in the saline agglutination test an open slide method is recommended for rapid work, in the albumin test a sandwich technique is used. Otherwise, tube techniques are recommended for both methods.

SALINE AGGLUTINATION TESTS

SLIDE TECHNIQUE. These tests are carried out on microscope slides. One volume, 0·03 ml. of antiserum is placed on the slide together with 1 volume of 5 per cent cell suspension and mixed with an applicator stick. The mixture is then gently rocked at room temperature for six minutes. These tests cannot conveniently be undertaken at 37° C. but are useful at 4° C. The result is then read with the naked eye, followed by microscopic examination under the low power of the

sensitivity is likely, depending on the specificity of the antigen and antibody.

e. Volume of reagents. The volume of reagent used will depend upon the nature of the test and whether it is carried out on a slide or in a tube. The unit volumes recommended are shown in Table 5. Normally each test will consist of one or more unit volumes of serum and one or more unit volumes of cell suspension.

TABLE 5. VOLUMES OF REAGENTS USED IN VARIOUS TESTS

Method	*Tube or slide*	*Unit volume of serum or cell suspension (ml.)*
Agglutination tests	Slide	0·03
Agglutination tests	Tube: 2×¼ inch	0·04
Large scale ABO grouping ..	Tube: 2×⅜ inch	0·08
Antiglobulin test	Tube: 3×½ inch	0·1
Haemolysin test	Tube: 2×⅜ inch	0·3

In tube agglutination tests read microscopically when rare sera are used, the unit volume can be reduced without affecting the efficacy of the test, but methods employing very small unit volumes, 0·01 ml. or less, are not considered valuable in routine work. If the unit volume in the anti-globulin test is reduced it will result in an unduly dilute cell suspension being placed on the slide in the second half of the test.

f. The temperature of the test. Many antigen-antibody reactions have an optimum temperature of activity. This temperature is the one at which that particular blood group test should be undertaken. The reagents should be at this temperature during the whole of the time they are in contact. Some reactions, such as the union of cold incomplete anti-H with red cells, act best at 0° C.; others, such as Rh antibodies, at 37° C. There are quite sharp differences in the titres of some antibodies at different temperatures (Table 6).

TABLE 6. EFFECT OF TEMPERATURE ON TITRE OF RH ANTIBODY, 5 PER CENT TEST CELL SUSPENSION

Temperature of test	*Titre*									*Score*
	2	4	8	16	32	64	128	256	*Control*	
37° C. ..	++++	++++	++++	+++	++	–	–	–	–	17
16° C. ..	+++	++	–	–	–	–	–	–	–	5
4° C. ..	+	w	–	–	–	–	–	–	–	1

g. The strength of the cell suspension. This affects the strength of agglutination observed. If the dose of cells is too great the antibody will be absorbed on to a large number of cells in small quantity on each, so that the agglutination may be quite feeble, or even indiscernible. If a thinner suspension is used then the antibody is absorbed in greater quantity on to each cell and the result is much better. It is necessary to strike a balance between too few cells which make the technique difficult, and too many cells (Table 7). Generally 3-5 per cent suspensions are used.

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Thickness of suspension	*Titre*									*Score*
	2	4	8	16	32	64	128	256	*Control*	
%										
2	+++++	++++	+++	++	++	+	–	–	–	17
5	+++++	++++	+++	++	+	w	–	–	–	15
10	++++	+++	++	+	–	–	–	–	–	10
20	+++	++	+	+	–	–	–	–	–	7

h. The presence of additional substances during the performance of the test. These may be necessary for the antigen-antibody reaction to occur, or more commonly, for it to be detected; e.g. complement in the case of cold incomplete anti-H and albumin for the final agglutination reaction in the case of certain incomplete antibodies.

Saline and Serum-albumin Agglutination Tests

The saline and serum-albumin agglutination tests are similar, but differ in that in the saline agglutination test the red cells are suspended in isotonic saline and the antiserum is diluted in this fluid. In the serum-[illegible] [illegible] [illegible] agglutination test an open slide method is recommended for rapid work, in the albumin test a sandwich technique is used. Otherwise, tube techniques are recommended for both methods.

SALINE AGGLUTINATION TESTS

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microscope. When reading the result, especially with the weaker agglutination reactions, examine a moving film (*vide infra*). Not only does this make the reading of the test easier, but assists in distinguishing true from false positive results. Control tests should be included to ensure:

1. that the cell suspension on its own is free from agglutination;
2. that the specific antigen-antibody reaction is taking place, both positive and negative controls being included.

SALINE AGGLUTINATION TUBE TECHNIQUE. Agglutination tubes, size $2 \times \frac{1}{4}$ inch, are usually employed for this method. 0·04 ml. of antiserum is introduced into the tube using a marked Pasteur pipette, and an equal volume of a 3 per cent suspension of red cells in saline is added.

The tube is tapped to mix the reagents, which are treated at the appropriate temperature by various means according to the antigens and antibodies involved and the circumstances of the test. These are:

(*a*) Sedimentation alone for $1\frac{1}{2}$–2 hours.
(*b*) Sedimentation for 30–45 minutes (10–15 minutes in Rh typing by the centrifuge technique, p. 184), followed by slow speed centrifugation, 500–1,000 r.p.m. for 2–3 minutes, e.g. Lewis blood grouping.
(*c*) Centrifugation at high speed for 2–3 minutes, immediately following the mixing of the reagents (p. 236).

In specifying the manner in which tests are to be centrifuged it is not the speed but the relative centrifugal force which must be specified. This is dependent on the diameter of the centrifuge as well as the speed. All the speeds given in this book refer to an 11 inch diameter centrifuge, so that 1,000 r.p.m. corresponds to an R.C.F. of $156 \times g$. The R.C.F. is calculated from the formula:

$$\text{R.C.F.} = 2{,}840 \times \text{R} \times (\text{r.p.m.})^2 \times 10^{-8}$$

where R = radius of the centrifuge in inches.

Slow speed centrifugation may always be used with advantage *following sedimentation to increase the strength of the agglutination in* these tests. It may, however, reveal the presence of antibodies not detected by sedimentation alone. Furthermore, if sedimentation has been at 37° C. cooling will occur during ordinary centrifugation.

Larger tubes ($2 \times \frac{3}{8}$ inch) than the $2 \times \frac{1}{4}$ inch recommended, are sometimes used with correspondingly larger volumes (0·08 ml.) of reagents, particularly where macroscopic reading only is to be employed (Chapter VI). These are unnecessary for general work as in any case the volume of reagents if all removed would flood the slide.

The test should be read microscopically. Using some antibodies, such as anti-A and anti-B, the agglutinates are very firm and the test may be read by tapping the tube. With the majority of antibodies,

e.g. anti-D saline agglutinins, the agglutinates are fragile and will not withstand shaking. A pipette should be introduced to the bottom of the tube and the cell deposit carefully removed and lightly spread out on a microscope slide. The tip of the pipette must be cleanly cut off at a right angle to the shaft and the whole of the contents of the tube must be gently pipetted on to a slide. The resulting drop of fluid should then be spread out either by tilting the slide or by drawing the drop over the surface of the slide with the pipette by surface tension (Fig. 1). One should avoid placing the pipette flat on the slide and spreading the drop with a "squashing" action. The slide should then be placed on the microscope stage, tilted, placed flat again, and the resultant moving field examined. It is much better to examine a moving field than a stationary one, though some Lewis agglutinations "reverse" whilst the field is moving and, in these cases, the appearance seen when the field is first examined should be taken as the correct reading. It is important to pipette the whole of the contents of the tube on to the slide and to examine all parts of the field, since agglutination may only occur in one area. With a cell mixture, for example, it might happen that some of the cells would agglutinate first and then sink to the bottom of the tube, where they would later be covered by a layer of unagglutinated cells, so that agglutination was only present in one part of the field.

Control tests are put up as for the slide agglutination test.

SALINE AGGLUTINATION TESTS—LAYERING TECHNIQUE. The cell suspensions and antisera are as described previously. One volume of 0·04 ml. of red cells is introduced into a tube and spun at 4,000 r.p.m. for 2–3 minutes. An equal volume of the antiserum, or a dilution of it, is then gently run down the side of the tube so as to be layered on the top of the packed cells without disturbing them. The tube and its contents are incubated at the appropriate temperature, usually 37° C., for 1½ hours. The results are read as stated above. By this means saline agglutinins can be detected in some sera in which they were not thought to exist, e.g. anti-Kell sera (Table 8).

TABLE 8. DEMONSTRATION OF SALINE AGGLUTININS IN ANTI-KELL SERUM USING A LAYERING TECHNIQUE

Method	*Titre*							
	1	2	4	8	16	32	64	*Control*
Sedimentation, 2 hrs. 37° C.	—	—	—	—	—	—	—	—
Layering technique	++++	++++	++	—	—	—	—	—

RESULTS OF AGGLUTINATION TESTS. These are illustrated in Plates III and IV.

Score		
5	+++++ (C)	= Massive clumps of cells with very few free cells, or one solid mass of cells, when the tube is tapped. C = complete.
4	++++ (V)	= Strong, visual, macroscopic result when the tube is tapped, but not a solid clump; the clump breaks up. Large masses of cells with only a few free cells. V = visual.
3	+++	= Result visible macroscopically in a tube or on a slide; under the microscope, many large clumps of cells with more free cells.
2	++	= Barely visible macroscopically in the tube or on the slide; under the microscope many clumps of ten to twenty cells with many free cells.
1	+	= Not visible macroscopically; under the microscope many clumps of about five-six cells with many free cells.
0	w	= Microscopically, many clumps of two-four cells with many free cells.
0	—	= Microscopic examination of the film shows free cells evenly flowing under the microscope.

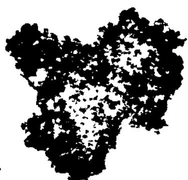
THE VALUE OF THE TEST. Many blood group antibodies have been reported from time to time to be active in saline. It is a simple and rapid technique and relatively free from difficulties of interpretation. Some antibodies, such as anti-A and anti-B, are most active in saline.

When antibodies such as Rh antibodies can be used by more than one technique, the advantage of saline agglutination methods is the clear-cut positive and negative results obtained. The use of centrifugation is advantageous; false positive aggregates of cells, or comets, are readily recognized as such.

SERUM-ALBUMIN AGGLUTINATION TESTS

Thirty per cent bovine albumin (Armour's) is employed and is stored at 4° C. The human serum is from a group AB donor free from all blood group antibodies and without rouleaux forming properties. The serum-albumin mixture consists of equal parts of 30 per cent bovine albumin and human serum. The red cell suspension is a 5 per cent suspension of packed cells in the serum-albumin mixture.

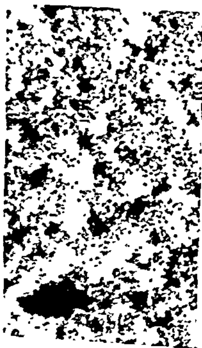
The test is carried out in agglutination tubes, $2 \times \frac{1}{4}$ inch. One volume (0·04 ml.) of antiserum is placed in the tube and to this is added one volume of cells suspended in serum-albumin. Titrations are prepared as described on p. 47. All the tubes are tapped to mix the contents and placed in a water bath at 37° C. or in previously warmed racks in the incubator at 37° C. They are allowed to sediment under gravity for a period of 2 hours. If a more rapid test is desired the tubes may be incubated for 45 minutes and gently spun at 500 r.p.m. for 2–3 minutes before the test is read. Control tests are employed and care should be taken to ensure that:



a +++++ (C).



b. ++++ (V).



c. +++.



d. ++.

PLATE. III. Strengths of agglutination. See p. 34. ×220

RESULTS OF AGGLUTINATION TESTS. These are illustrated in Plates III and IV.

Score		
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3	+++	= Result visible macroscopically in a tube or on a slide; under the microscope, many large clumps of cells with more free cells.
2	++	= Barely visible macroscopically in the tube or on the slide; under the microscope many clumps of ten to twenty cells with many free cells.
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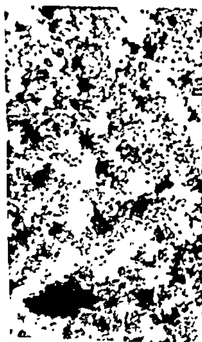
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a + + + + + (C).



b. + + + + (V)



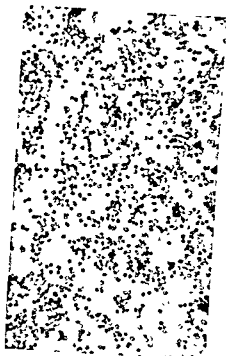
c + + +.



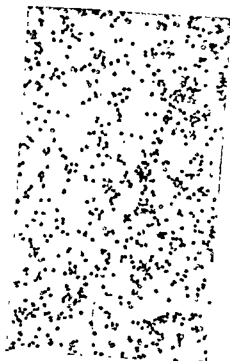
d. + +.

PLATE. III Strengths of agglutination. See p. 34. ×220

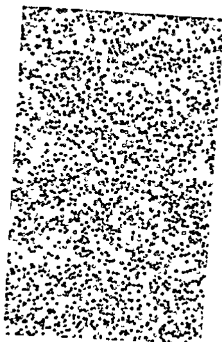
PLATE IV. Strengths of agglutination (*contd*). ×220



a +



b. w.



c Negative.

i. The specific antigen-antibody reaction is occurring, using positive and negative control cells.
ii. The cells are free from agglutination, a volume of group AB serum being substituted for the antiserum.
iii. The patient's own cells suspended in serum albumin, together with the patient's own serum, are unagglutinated (*vide infra*).

The control tubes are treated in the same way as the actual test tubes.

The results are read as described under saline agglutination tube methods (p. 33).

FALSE POSITIVE AND FALSE NEGATIVE RESULTS are dealt with in Chapter V, but the following points should be specially noted.

False positive results in this test are much more troublesome than in the saline agglutination test. Comets or false positive aggregates, especially in centrifuged deposits, are not readily distinguished from true agglutinates and do not rapidly disperse. Examination of a moving film assists. The initial reading may be followed by the addition of a drop of saline, rocking the slide and re-reading. Experience is needed to interpret centrifuged deposits in the albumin test.

Batches of albumin vary from one to another and even the best serum albumin mixtures do give weak false positive results from time to time in the control tests. It may be necessary to repeat the test using another batch of albumin or serum from a different group AB donor. Rouleaux formation may be enhanced.

An albumin auto-agglutinating property of serum has been described (Weiner, Tovey, Gillespie, Lewis and Holliday, 1956). Such a serum does not agglutinate saline suspended cells, but all albumin suspended cells, including the patient's own cells, are agglutinated at 4°, 22° and 37° C. Atypical antibodies were not present and this property could not be removed by absorption. It is a rare phenomenon, but a control tube of the patient's own cells in serum albumin with the patient's own serum is desirable.

False negative results. The liability to prozone makes the test in the form of a short titration to 8–16 of more value than a single tube test.

OBSERVATIONS ON THE [illegible]

[illegible] detection of incomplete antibodies than the widely employed 20 per cent bovine albumin introduced by Diamond and Abelson (1945), or human plasma alone, used by Wiener (1945). Extensive tests led to the conclusion that a mixture of equal parts of 30 per cent bovine albumin and of whole serum was the best diluting fluid (Stratton

and Dimond, 1955). Table 9 shows the result of titrating an Rh antibody using the various diluents.

TABLE 9. ANTI-D SERUM TITRATED IN VARIOUS DILUENTS AT 37° C. R_1R_1 TEST CELLS

Diluent	*Titre*									
	1	2	4	8	16	32	64	128	256	*Control*
Saline ..	−	−	−	−	−	−	−	−	−	−
AB serum ..	+++	++	+	−	−	−	−	−	−	−
20% bovine albumin ..	++++	++	+	−	−	−	−	−	−	−
Serum albumin ..	++++	++++	+++	+++	++	+	+	−	−	−

From time to time other substances have been suggested as a substitute for bovine albumin or human serum or mixtures of these. Various other viscous fluids will undoubtedly act in a manner similar to these reagents. Dextran, gum acacia, methyl cellulose, polyvinylpyrrolidone, and so on, have been recommended. The disadvantage of these substances is that either they fail to potentiate the agglutination in the way in which serum-albumin does, or the control tests are much more difficult to interpret; that is to say, they tend to cause a higher proportion of false positive results. None of these substances are useful substitutes for the serum-albumin mixture.

THE VALUE OF THE TEST. The albumin test is widely used for routine purposes and is superior to the saline agglutination test for the detection of Rh antibodies. Nevertheless, many Rh antibodies, as commonly found in maternal sera during the antenatal period, especially the early antenatal period, are not detected by the serum albumin test.

Other Rh antibodies, such as anti-c are found to be much more active in serum albumin than they are in 20 per cent bovine albumin. For example, one anti-c antibody with a titre of 64 in 20 per cent. albumin had a titre of 256 in serum albumin. The saline titre was 4. Similarly, an anti-E antibody gave negative reactions in saline, a titre of 4 in albumin, and a titre of 16 in serum albumin. Occasionally, examples of anti-E are found which are negative using the antiglobulin test but give positive results using the albumin test or enzyme treated red cells. Similarly, occasional anti-D antibodies during the early months of pregnancy may react in the same manner. Stratton and Renton (1949) showed that D^u cells of low grade are not agglutinated by incomplete Rh antibodies when the red cells are suspended in 20 per cent bovine albumin diluent. The substitution of serum albumin enables some of

these to be detected. Certain low-grade D^u cells otherwise not detectable using an albumin technique can be detected if the diluent is serum albumin, but a prozone may occur. This is illustrated in Table 10 (Stratton and Dimond, 1955).

TABLE 10. TITRATIONS AT 37° C. PERFORMED TO INVESTIGATE THE BEHAVIOUR OF D^u CELLS IN VARIOUS DILUENTS

Serum	*Cells*	*Diluent*	*Titre*									
			1	2	4	8	16	32	64	128	256	Control
Incomplete anti-D (i)	(*a*) $R_1^u r$	20% albumin	—	—	—	—	—	—	—	—	—	—
		Serum albumin	w	++	+++	++	+	w	—	—	—	—
		Saline	—	—	—	—	—	—	—	—	—	—
	(*b*) Normal $R_0 r$	20% albumin	++++	+++	+++	+++	++	+	—	—	—	—
		Serum albumin	++++	++++	+++	+++	+++	++	+	w	—	—
		Saline	—	—	—	—	—	—	—	—	—	—
	(*c*) $R_2^u r$	20% albumin	w	w	+	+	—	—	—	—	—	—
		Serum albumin	++	+++	+++	++	++	+	—	—	—	—
		Saline	—	—	—	—	—	—	—	—	—	—
Incomplete anti-D (ii)	(*d*) $R_2^u r$	20% albumin	—	—	—	—	—	—	—	—	—	—
		Serum albumin	+	+	+	—	—	—	—	—	—	—
		Saline	—	—	—	—	—	—	—	—	—	—

One of the disadvantages of the albumin technique is its liability to show zoning phenomena. Serum albumin materially reduces the risk of prozone occurring during the titration of sera but, *nevertheless, it* does not make it safe to use a one tube albumin test. Table 11 illustrates this. In a one tube technique a stronger result would be obtained if zoning does occur using serum albumin than using 20 per cent. bovine albumin. The technique of the test also has a bearing on the occurence of zoning, and if a centrifugation technique is used there is no doubt that the tendency to zoning is reduced and prozones may, under certain circumstances, be abolished. The disadvantages of centrifugation in interpretation of the results, however, make this difficult to use as a general method.

A number of antibodies are not detected by this technique, anti-Fy^a, anti-Jk^a and some examples of anti-S. In some instances the results are no better than using saline agglutination methods, e.g. anti-Le^a and anti-Le^b; indeed, in the case of certain anti-A and anti-B antisera the results may be somewhat worse.

TABLE 11. THREE SERA SHOWING ZONING IN 20 PER CENT ALBUMIN TITRATED AGAINST VARIOUS CELLS IN DIFFERENT DILUENTS AT 37° C.

Serum	Cells	Diluent	Titre 1	2	4	8	16	32	64	128	256	512	1,024	2,048	4,096	8,192	16,000	32,000	64,000	Control
Incomplete anti-C +D	R_1r	20% albumin	++	w	−	−	−	w	++	+++	+++	+++	++	+	w	−	−	−	−	−
		Serum albumin	+++	++	++	+++	+++	+++	++++	++++	+++++	+++++	++++	+++	+++	++	++	+	−	−
		Saline	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
	R_1R_1	20% albumin	+++++	+++++	++++	+++	++	+	+++	+++	++	++	+	w	−	−	−	−	−	−
		Serum albumin	+++++	+++++	+++++	++++	++++	++++	++++	++++	++++	++++	++++	+++	+++	++	++	+	−	−
		Saline	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Incomplete anti-C +D	R_2R_2	20% albumin	w	−	−	−	+	++	+++	+++	+++	++	+	w	−	−	−	−	−	−
		Serum albumin	+++	+++	++++	++++	++++	++++	++++	+++++	+++++	++++	++++	+++	+++	++	+	−	−	−
		Saline	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Incomplete anti-D	R_1R_1	20% albumin	w	++	+++	++++	+++	+++	++	w	−	−	−	−	−	−	−	−	−	−
		Serum albumin	+++	++++	+++++	+++++	+++++	++++	+++	++	+	w	−	−	−	−	−	−	−	−
		Saline	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−

A whole serum diluent is occasionally more valuable than serum-albumin, e.g. incomplete anti-A, anti-B, (Witebsky 1947). Some examples of anti-Kell may not be detectable in 20 per cent albumin at all, but only in serum albumin and preferentially in whole serum (Table 12 and p. 290).

TABLE 12. TITRATION OF AN ANTI-KELL SERUM WITH KELL-POSITIVE CELLS AT 37° C. IN VARIOUS DILUENTS

Diluent	*Titre*					
	1	2	4	8	16	*Control*
20% albumin	–	–	–	–	–	–
Serum albumin	++	+	w	–	–	–
Saline..	–	–	–	–	–	–
AB serum	+++	++	++	+	–	–

Serum albumin tests will detect anti-P or anti-M at 16° C. and occasionally at 37° C.

The first point that emerges from this is that serum albumin should be substituted for 20 per cent bovine albumin in this test. Secondly, that the test is superior in certain respects to the saline agglutination test; namely, that it will detect a greater proportion of Rh antibodies owing to the fact that it will detect many incomplete Rh antibodies. Thirdly, a higher proportion of D^u positive persons can be detected than if saline agglutination tests are used. Fourthly, certain antibodies not readily demonstrated by other means will be detected and it is always desirable therefore to include it for the identification of atypical antibodies in sera. On the debit side are its liability to prozones and its failure to detect many examples of atypical antibodies. This has necessitated the substitution of alternative methods in routine procedures, such as antenatal testing and cross-matching.

All these observations refer to tube techniques. This test has always been deservedly popular for rapid Rh typing and we previously used the tube method, but recently have successfully used a sandwich method (Stratton, 1955), (p. 185). Whereas the open slide method of Diamond (1945) is best used with selected high titre Rh antisera, the sandwich method can be used with relatively weaker ones. The method may also be used for the rapid selection of persons of particular Rh groups (p. 233).

Agglutination Tests with Enzyme Treated Red Cells

Morton and Pickles (1947) described the use of enzyme treated test cells for the detection of incomplete Rh antibodies. Morton and

Pickles (1951) subsequently described the treatment of red cells with trypsin for their use in this manner. Kuhns and Bailey (1950) employed papain to treat the red cells. Rosenfield and Vogel (1951) used trypsin and papain treated test cells. They showed that using trypsinized cells there was enhancement of the activity of anti-P and anti-Lewis antibodies as well as of Rh antibodies, but destruction of M, N and Fy^a antigens. They also described the occurrence of autoagglutinins to enzyme treated cells.

Here it is proposed to describe the method that we have employed for some years (Stratton 1953), in which papain was used together with an activator, cysteine. This was the first occasion on which papain had been used in combination with the activator and it enabled the treated cells to be used for the detection of antibodies on a slide at room temperature.

Preparation of Treated Cells. The red cells are collected in A.C.D. mixture, 4 volumes of whole blood to 1 volume of anticoagulant. If the blood is freshly collected the mixture should be left for an hour before the cells are treated. The cells are then washed three times in normal saline. One volume of the papain buffer mixture consisting of equal parts of papain and buffer solution, together with 1 volume of activator, is mixed with 1 volume of washed packed red cells. The mixture is incubated in the water bath at 37° C. for 10 minutes. This is then centrifuged and the supernatant removed. The treated cells are then washed twice with normal saline. A 5 per cent suspension is used in the test. The various solutions are prepared as follows:

Enzyme solution:	Papain powder (B.D.H)	gm 1
	Normal saline..	ml. 100
Buffer:	Disodium hydrogen phosphate	
	($Na_2HPO_4 12H_2O$)	gm. 3 6
	Water	ml. 100
Activator:	L. Cysteine hydrochloride	gm. 0 2
	Saline	ml. 100

The papain solution is prepared by shaking the powder with saline, spinning it down and removing the clear supernatant liquid. This is divided into 5 ml. quantities and preserved at −25° C. It should be renewed at least every four weeks. The buffer solution is kept in the refrigerator and should be sterile. The cysteine is freshly prepared since it rapidly becomes changed to cystine. 2–3 ml. of packed cells are normally treated at one time. The papainized red cells can be kept in the refrigerator for three days and are normally prepared twice a week.

Standardization of the Papainized Cells. A volume of a 5 per cent suspension of the cells is mixed on a slide with each of several normal sera diluted 1 in 2 to ensure that the cells so prepared are not

spontaneously agglutinable. If this test is negative the red cells are then further examined for sensitivity. This is done by titrating them with a standard incomplete Rh antibody in order to ensure, on each occasion, that the cells are equally strongly papainated. The titres of this standard antibody using various methods are shown in Table 13.

TABLE 13. TITRATION OF STANDARD ANTIBODY USING VARIOUS TESTS

Nature of test	*Titre of standard anti-D serum*									
	2	4	8	16	32	64	128	256	512	Com-pool
Albumin agglutination ..	+++++	+++++	+++	+	−	−	−	−	±	−
Coombs test ..	+++++	+++++	+++++	+++++	+++	++	−	−	−	−
Papainized cell test	+++++	+++++	+++++	++++	++++	+++	++	+	−	−

A dilution of 1 in 100 of this particular Rh antibody is used and 1 volume of this is added to 1 volume of a 5 per cent suspension of the cells on a slide, mixed and rocked for 6 minutes. A positive visible result, ++ to +++, should be obtained.

Rh negative cells may be papainated in the same way as Rh positive ones and are standardized by using an anti-c antiserum. Table 14 shows the results obtained by titrating such a serum with papainated red cells.

TABLE 14. COMPARISON OF STANDARD ANTISERA

Test serum	*Papainized test cell*	*Titre*										
		2	4	8	16	32	64	128	256	512	1024	Com-pool
Anti-D (standard)	R_1r	+++++	+++++	+++++	+++++	++++	+++	++	+	−	−	−
Anti-c	R_1r	+++++	+++++	+++++	+++++	+++++	+++++	++++	++++	++	−	−
	rr	+++++	+++++	+++++	+++++	+++++	+++++	++++	++++	++	−	−

Any sera may similarly be used at greater or lesser dilution. When red cells are papainated for the purpose of carrying out other blood group tests, they are papainated and tested as described above. If they give satisfactory [illegible]

[illegible]

TECHNIQUE OF THE TEST. The test is performed on a microscope slide at room temperature. Several tests may be performed by one worker at the same time and the slides are then laid out on a larger opal

tile. One volume (0·03 ml.) of the serum under test (diluted to 1 in 2 in normal saline) is placed on the slide. One volume (0·03 ml.) of a 5 per cent suspension of the papainized test cells is added and the reagents are mixed together. Thus the final dilution of the serum is 1 in 4. Alternatively, 1 volume of whole serum is placed on the slide, together with an equal volume of saline and 2 volumes of a 5 per cent suspension of papainized red cells. The slides are gently rocked for a period of 6 minutes. The results are read with the naked eye and those which appear negative are examined under the low power of the microscope. The results are recorded and scored as previously stated (p. 34). Control tests are put up on each occasion consisting of papainized cells and normal serum diluted 1 in 2. This test should be negative. Autoantibodies active against papainized red cells occur only rarely. A certain number of these autoantibodies are also active against papainized red cells at 37° C. (Table 18, p. 46).

If positive tests are obtained on a slide at room temperature it is often advantageous to know whether the antibody is active against papainized red cells at 37° C. Rh antibodies are active at 37° C. as well as at room temperature, but a number of positive results occur at room temperature due to other antibodies or to feeble autoantibodies which are not active at 37° C.

This confirmatory test, expecially in the case of Rh antibodies, is conveniently and rapidly done in capillaries (0·75 to 1 mm. bore). The *mixture of cells serum and saline is drawn up into a capillary which is* supported at an angle of 60° in a piece of plasticine at 37° C. for 30 minutes, either in the water bath or on a specially constructed warm viewing box. The result is read with a hand lens. In a positive result the cell column is striated and broken up; in a negative result no such striations are seen and the cell column is represented by a thin continuous line. Sometimes, if left too long, the cells will be found to have settled completely to the bottom of the tube, in which case the tube should be inverted or the test repeated.

A tube technique may be used similar to that described for the saline agglutination, but substituting papainized for normal red cells. This is most useful for tests at 16° C.

Rh antibodies active at 37° C. are more readily detected when mixed with the papainized cells in capillaries than in tubes.

The test is designed for the papainization of cells collected into A.C.D. as described (p. 19). If different proportions of blood to anticoagulant, or different anticoagulants are used, different degrees of papainization may be obtained.

Cells collected as defibrinated or clotted blood, or a few drops of cells collected in a large volume of anticoagulant, may be papainated to a different degree from those collected into A.C.D. (p. 19). It may be that citrate ions rapidly entering the cells emerge only slowly,

affecting their buffering power, or that citrate damages cell permeability (Keilin and Mann, 1941; Holton, 1952). Nevertheless, these different effects do not seem to be wholly accounted for by differences in pH and it is desirable to have a standard method of papainization. The final mixture during papainization should be pH 6.8–6.9.

To change red cells to the required degree so that they would agglutinate in the presence of, for example, weak Rh antibodies on a slide at room temperature, it was necessary to employ the enzyme papain together with an activator. Papain alone was not satisfactory nor was trypsin. In the crude enzyme preparation itself there is probably a naturally occurring activator, glutathione. Other activators are sodium cyanide, cysteine hydrochloride and B.A.L. (2 : 3 dimercapto-propanol). Of all these activators, B.A.L. is probably the best, and activates the papain very strongly indeed, but cysteine hydrochloride is the best for routine work. The use of B.A.L. may change the cells to such an extent that more false positive results than desirable occur.

TABLE 15. COMPARISON OF METHODS OF ENZYME TREATMENT OF RED CELLS

Treatment of test cell	Titre										
	2	4	8	16	32	64	128	256	512	1024	Control
Papain + activator ..	+++++	+++++	+++++	++++	+++	+++	++	+	—	—	—
Papain .. .	+++++	+++++	++++	+++	++	+	—	—	—	—	—
Trypsin .	++++	+++	+	—	—	—	—	—	—	—	—

Test cell = R_1R_1. Test Serum = Standard anti D serum.

Table 15 shows a comparison of the methods of enzyme treatment of red cells carrying out the titrations on slides at room temperature. The use of an activator with the papain gives a higher titre with the antibody than with papain alone. The trypsinised red cells give a feeble result, but they are not intended to be used by this particular technique.

Papain inhibitors are present in normal sera and consequently the cells must be washed before they are treated with the enzyme activator mixture. Different cell samples vary in the extent to which they are changed when treated in the same manner. Standardization is, therefore, necessary and a diluted human anti-Rh serum is used. It is possible to use a rabbit anti-papain red cell agglutinating serum, but the human serum is preferred.

The enzyme solution should be stored frozen solid and the cysteine made up freshly and added to the papain solution just prior to the test, together with the buffer. Cysteine is most stable as the hydrochloride, and neutral solutions tend to change to cystine.

Methods involving the mixing of papain and activator with antiserum

tile. One volume (0 03 ml.) of the serum under test (diluted to 1 in 2 in normal saline) is placed on the slide. One volume (0·03 ml.) of a 5 per cent suspension of the papainized test cells is added and the reagents are mixed together. Thus the final dilution of the serum is 1 in 4. Alternatively, 1 volume of whole serum is placed on the slide, together with an equal volume of saline and 2 volumes of a 5 per cent suspension of papainized red cells. The slides are gently rocked for a period of 6 minutes. The results are read with the naked eye and those which appear negative are examined under the low power of the microscope. The results are recorded and scored as previously stated (p. 34). Control tests are put up on each occasion consisting of papainized cells and normal serum diluted 1 in 2. This test should be negative. Autoantibodies active against papainized red cells occur only rarely. A certain number of these autoantibodies are also active against papainized red cells at 37° C. (Table 18, p. 46).

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treat with papain. In this case a fresh sample of different red cells should be tried. If the red cells are not changed to a sufficient degree, alteration of the time and attention to the temperature of incubation will often put it right.

ii. Technical errors in the performance of the test comprise failure to keep the cells and serum on the slide gently rocked for the appropriate time and failure to detect the weaker positive results.

If strong autoantibodies are present in a serum they may obscure the detection of another antibody, and an alternative test will have to be used or the serum absorbed with its own enzyme treated cells and retested.

VALUE AND IMPORTANCE OF THE TEST. The slide technique was introduced as a rapid screening test for the detection of Rh antibodies. It is used in antenatal testing (p. 199), in screening the sera of Rh negative donors, and is an ancillary test in the identification of antibodies.

It is the most sensitive technique for the detection of Rh antibodies (Table 16 and Table 18).

TABLE 16. TESTS OF SERUM DI-, WHICH CONTAINS WEAK RH ANTIBODIES

Saline agglutination	*Albumin agglutination*	*Routine Coombs test with mixed gp. O, Rh pos. cells*	*Special Coombs tests with selected cells*										*Papainized Rh positive cells*
			R_1r	R_1R_1	R_2r	R_1R_2	R_2R_2	R_0r	$R'r$	rr	rr	rr	
—	—	—	+	++	+	—	—	+	—	—	—	—	+++

The test, either on slides or in tubes at 16° C., is a very good method for the detection of anti-Le^a, anti-Le^b and anti-P antibodies and the titres and avidities of these antibodies are enhanced by using papainized in preference to normal cells (Tables 89 and 104, pp. 287 and 297).

TABLE 17. TYPICAL RESULTS USING THE ENZYME SLIDE TEST AT ROOM TEMPERATURE AND CAPILLARY TEST AT 37° C.

Antibody	*Slide test 20° C.*	*Capillary test 37° C.*
Anti-P	++++	Neg.
Anti-Le^a	++++	Pos. or Neg.
Anti-Le^b	+++	Pos. or Neg.
Autoagglutinin	+++	Neg.
Anti-H	++	Neg.
Weak anti-D	+++	Pos.
Strong anti-D	+++++	Pos.

and cells to be tested, and carrying out the whole in a single stage, have been described (Löw, 1955). It is known that sera contain papain inhibitors which, in such a test, must make the activity of the enzyme variable even if they are not sufficiently strong to inhibit it completely. Secondly, under certain circumstances, some antibodies are digested by papain in a short period of time and the activity of some weak ones will thus be diminished, if not destroyed, by this procedure. Thirdly, such methods may involve the use of inadequate buffers or of unstable solutions of neutral cysteine. Both false positive and false negative results may be encountered.

FALSE POSITIVE AND FALSE NEGATIVE RESULTS. The general features of false positive and false negative results, described in Chapter V, apply in the case of this test. There are certain special difficulties.

False positive results may be due to:

i. Spontaneous agglutination of the papainated cells.
ii. Failure to dilute the patient's serum at least 1 in 2.
iii. Other difficulties described in Chapter V, e.g. infected serum, rouleaux forming properties of serum and autoantibodies.

i. Spontaneous agglutination of the papain cells will sometimes occur if they are over papainated, and this will be detected by the control test which should always be included. If the cells act in this way they should be discarded and a new batch prepared.

ii. There appears to be some factor in certain normal undiluted sera capable of agglutinating papainized red cells. This is the reason for diluting sera 1 in 2 before testing with papainized cells.

iii. Rouleaux forming properties are not such a disadvantage using this technique because of the initial dilution of the patient's serum to 1 in 2, and this is of particular advantage when testing maternal sera where rouleaux forming properties are not uncommon. It is only when using sera containing very potent rouleaux forming properties (p. 96) that further dilution is required. Autoantibodies against enzyme treated cells rarely occur in sera and they are mainly active at room temperature. Occasionally, autoantibodies occur active at 37° C. against enzyme treated cells but not against normal cells (Table 18).

False negative results may be due to:

i. Inadequate treatment of the red cells.
ii. Technical errors.

i. Failure to papainate the red cells to the required extent should be detected when the standardization test is used. Failure may be due to the pH being wrong or to inactivity of one of the reagents. Cell samples are occasionally found which, for reasons unknown, are difficult to

to unite with an antigen. It is a measure of the grasping power of the [illegible] which an antibody, or a [illegible] or reacting, or uniting with homologous red cells.

TECHNIQUE OF TITRATION. If the antibody under test acts in saline or in serum albumin, or in serum, serial dilutions of the antibody are made in these media (but see below). If it reacts only by the antiglobulin test, serial dilutions of the serum are made and an antiglobulin test done on each dilution. In either case, a row of tubes is set up and numbered with a control tube at the end. Into each tube is placed one volume of the diluent, 0·04 ml. in the case of the agglutination test, and 0·2 ml. in the case of the antiglobulin test. An equal volume of the serum which is being titrated is added to the first tube and, using a marked Pasteur pipette, these two are mixed together by squeezing gently in and out of the pipette six or seven times. One volume is then transferred to the second tube and the process repeated. A volume is discarded from the last tube. This will give doubling dilutions of serum in the appropriate medium, i.e. 1 in 2, 1 in 4, 1 in 8, and so on. One volume of the standard dose of test cells in the appropriate diluent is now added to each tube and the mixtures tapped and incubated at the optimum temperature for 40 minutes to 2 hours, according to the diluent and test being employed. Each tube is then read as described previously for the particular test and the results scored accordingly. It is desirable that two negative results should conclude the titration.

When titrations are made in serum albumin, the higher tubes of the titration contain more serum albumin than the lower ones. It is an advantage to dilute the serum in serum and add the cells suspended in serum albumin, since sedimentation in high concentrations of albumin is unduly slow.

The control tube is a cell suspension control consisting of one vol. of diluent together with one vol. of cell suspension. This is the "control" referred to in many tables detailing titrations.

The above method of titration is subject to error by reason of:

(*a*) The fact that the use of a marked Pasteur pipette is an inaccurate way of measuring volumes.
(*b*) The use of doubling dilutions is an inaccurate way of making dilutions.
(*c*) Using a single pipette for the whole titration involves the risk of carrying over more concentrated serum into the more dilute parts of the titration.

This last difficulty is overcome by using a fresh pipette for the transfer of each volume of serum from one tube to the succeeding higher dilution, or by washing out the pipette after making each dilution.

Anti-H and anti-O are active against papainized cells at 16° C. Table 18 shows the results of a month's work using the papain slide technique for screening antenatal sera and Table 17 typical results using the test.

TABLE 18. RESULTS OF A MONTH'S TESTS ON MATERNAL SERA SHOWING ANTIBODIES DETECTED BY PAPAIN SLIDE TEST AND WHOSE IDENTITY WAS SUBSEQUENTLY CONFIRMED

Antibodies	*Number detected: Papain slide test, room temperature*
Anti-Rh	85
Anti-P	24
Anti-H/O	11
Anti-Le^a } Anti-Le^b }	4
Autoagglutinin*	9

* 3 positive at 37° C. Total number of samples tested: 1,346.

We have no evidence to suggest that any Rh antibodies have been missed using this slide technique at room temperature with the serum diluted 1 in 2, and the advantage of a low temperature test is that other potentially dangerous antibodies are found, such as anti-Le^a.

The test is free from prozone phenomena and false positive results occur infrequently. Table 18 shows that of 1,300 sera examined, nine autoagglutinins were detected, three of which were active at 37° C. The slide technique may detect anti-K, but not anti-Lu^a. The antigens, M, N and Fy^a appear to be destroyed by the enzyme activity.

In conclusion, it is interesting to observe that the immediate agglutination and reversal phenomenon (Rosenthal and Schwartz, 1951) between normal sera and trypsinized red cells does not occur using papainized ones. Perhaps this is because it is prevented by cysteine, which is one of the substances known to inhibit the phenomenon with trypsinized cells.

Quantitation of Antibody

Quantity of antibody in a serum may be measured in two ways:

1. Titration.
2. Avidity determinations.

Titration of an antibody is a method of determining the highest dilution at which th[illegible] fixed dose of test cells. [illegible]
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to unite with an antigen. It is a measure of the grasping power of the antibody; it measures the extent and speed with which an antibody, or a dilution of antibody, is capable of agglutinating or reacting, or uniting with homologous red cells.

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Anti-H and anti-O are active against papainized cells at 16° C. Table 18 shows the results of a month's work using the papain slide technique for screening antenatal sera and Table 17 typical results using the test.

TABLE 18. RESULTS OF A MONTH'S TESTS ON MATERNAL SERA SHOWING ANTIBODIES DETECTED BY PAPAIN SLIDE TEST AND WHOSE IDENTITY WAS SUBSEQUENTLY CONFIRMED

Antibodies	*Number detected: Papain slide test, room temperature*
Anti-Rh	85
Anti-P	24
Anti-H/O	11
Anti-Le[a] } Anti-Le[b] }	4
Autoagglutinin*	9

* 3 positive at 37° C. Total number of samples tested: 1,346.

We have no evidence to suggest that any Rh antibodies have been missed using this slide technique at room temperature with the serum diluted 1 in 2, and the advantage of a low temperature test is that other potentially dangerous antibodies are found, such as anti-Le[a].

The test is free from prozone phenomena and false positive results occur infrequently. Table 18 shows that of 1,300 sera examined, nine autoagglutinins were detected, three of which were active at 37° C. The slide technique may detect anti-K, but not anti-Lu[a]. The antigens, M, N and Fy[a] appear to be destroyed by the enzyme activity.

In conclusion, it is interesting to observe that the immediate agglutination and reversal phenomenon (Rosenthal and Schwartz, 1951) between normal sera and trypsinized red cells does not occur using papainized ones. Perhaps this is because it is prevented by cysteine, which is one of the substances known to inhibit the phenomenon with trypsinized cells.

Quantitation of Antibody

Quantity of antibody in a serum may be measured in two ways:

1. Titration.
2. Avidity determinations.

Titration of an antibody is a method of determining the highest dilution at which the antibody will give a positive (+) result with a fixed dose of test cells. The titre is the reciprocal of the highest dilution that will give this positive result. Avidity is a measure of the ability of an antibody

If a more accurate method is required, dilutions should be made separately and transferred with a separate pipette to each individual test tube; graduated pipettes are used throughout.

For ordinary routine work, the less accurate method is satisfactory. The essential thing is to be able to get consistent results and each individual must determine his or her error. This is done by titrating the same serum six or eight times in exactly the same way, using the same cells and noting the titre and score. It will usually be found that the method is accurate to ± one tube, and this is the extent, therefore, to which the titre of one serum may be compared with that of another.

AVIDITY DETERMINATION. Avidity tests with anti-A and anti-B sera are described on p. 113. Where antibodies do not agglutinate red cells on slides but only in tubes, a titration is made as above and scored as described on p. 34. The score of each tube in the titration is then added. In this way an estimate of the avidity of the serum is obtained. This is illustrated in Table 19.

TABLE 19. TITRATION OF FOUR ANTI-D SERA OF THE SAME TITRE BUT DIFFERENT AVIDITIES. R_1r TEST CELLS

	2	4	8	16	32	64	128	256	*Control*	*Titre*	*Score*
				Saline diluent							
Anti-D (i)	+++++	+++++	+++++	+++	++	+	–	–	–	64	21
Anti-D (ii)	+++	+++	++	++	+	+	–	–	–	64	12
				Serum albumin diluent							
Anti-D (iii)	+++++	+++++	+++++	++++	+++	++	–	–	–	64	24
Anti-D (iv)	++++	+++	+++	++	++	+	–	–	–	64	15

It will be observed that the sera have the same titre but different scores. These results of the scoring of the titration indicate that the grasping powers of serum (i) and serum (iii) are greater than those of sera (ii) or (iv) respectively. The former sera are more advantageous as blood grouping sera.

The score of a titration and the apparent avidity of the serum will vary according to the dose of homologous antigen on the test cell (p. 280). Consequently it is desirable to judge the avidity and value of a serum as a possible blood grouping serum using heterozygous and less strongly active erythrocytes.

References

Cahan, A. (1955). Fluid for shipping whole blood specimens. *J.A.M.A.*, 158, 971.

Chown, B., and Lewis, M. (1946). Further experience with the slanted capillary method for Rh typing of red blood cells. *Canad. Med. Ass. J.*, 55, 66.

CHAPTER IV

COMPLEMENT IN BLOOD GROUPING

Complement is a property of serum and consists of four main components. In Chapter I reference has been made to the two main types of complement, the haemolytic and conglutinating kinds, and to some of the properties of the various components. In this chapter it is proposed to describe a routine method for the titration of complement. For further information on complement, on more accurate titration methods, together with details for titration of haemolysin, complement components and their properties and use, the reader is referred to Kabat and Mayer (1948), as well as Pillemer (1943) and Osborn (1937). Whole complement (C') consists of four components, C'1, C'2, C'3 and C'4 (p. 11).

Complement Titration

Materials

Buffered saline, pH 7·4 (barbital or veronal buffer with additional magnesium and calcium) (Pillemer *et al.*, 1956).

Concentrated stock solution:

85·0 gm.	NaCl
5·75 „	5,5-diethylbarbituric acid
3·75 „	Sodium 5,5-diethylbarbiturate
5·0 ml.	M $MgCl_2$
1·5 „	M $CaCl_2$
2 litres	Distilled water

The ingredients are dissolved in 1,500 ml. of hot water and the volume made up to 2,000 ml.

This stock solution is stored at 4° C.; for use, 1 volume is added to 4 volumes of distilled water and freshly made each day.

Sensitized cells are prepared by washing sheep cells three times with buffer and making a 2·5 per cent suspension. The strength of this suspension is checked by haemoglobinometry and adjusted to a standard.

One volume of barbital buffer containing four minimal haemolytic doses of haemolysin is added to an equal volume of a 2·5 per cent suspension of red cells, mixed together and incubated at 37° C. for 30 minutes. The standard is so arranged that each 1 ml. dose of sensitized cells as used in the test contains $2{\cdot}25 \times 10^8$ red cells; the sensitized cells are prepared freshly each day.

Technique of the Test

The technique is similar to that of Pillemer *et al.* (1956). This is

Wiener, A. S. (1945). Conglutination test for Rh sensitization. *J. Lab. Clin. Med.*, 30, 662.

Wiener, A. S., and Hurst, Jane G. (1947). A new sensitive (direct) test for Rh blocking antibodies. *Exp. Med. Surg.*, 5, 285.

Wiener, A. S., Hurst, Jane G., and Sonn-Gordon, Eve B. (1947). Studies on the conglutination reaction, with special reference to the nature of conglutinin. *J. Exp. Med.*, 86, 267.

Witebsky, E., Rubin, M., Engasser, M., and Blum, L. (1947). Studies in erythroblastosis foetalis. II. Investigation on detection of sensitization of red blood cells of newborn infants with erythroblastosis foetalis. *J. Lab. and Clin. Med.*, 32, 1339.

1. Heating.
2. Change in pH.
3. Absorption by antigen-antibody aggregates.
4. Action of plasmin.
5. Removal of Ca^{++} and Mg^{++}.
6. Storage.

1. The normal way of inactivating complement is by heating at 56° C. for 30 minutes. The thermo-labile components, C′1 and C′2 are destroyed, leaving C′3 and C′4 in reduced titre. Heating at 50° C. for approximately 40 minutes may also render complement inactive; C′2 is destroyed (Ecker *et al.*, 1943).

2. The effect of pH change on complement has been studied by Seifter *et al.* (1943). They added various amounts of acid and alkali to human sera and allowed them to stand at room temperature for 1 hour. Haemolytic activity of whole serum was completely destroyed at pH 4·2 and pH 10. It seems likely that complement is completely active between pH 7 and pH 8·4.

3. Treatment of complement with antigen-antibody aggregates results in complete inactivation of the second and fourth components, C′2 and C′4, and partial inactivation of the first component, C′1, whereas there is little or no inactivation of C′3. We have used egg albumin/rabbit anti-egg albumin precipitate for decomplementing sera. It is interesting to note that Weigle and Maurer (1957) have shown that although such antigen-antibody precipitates may be completely saturated with complement, they still remove haemolytic activity from fresh serum.

4. Plasmin prepared by the addition of streptokinase to normal serum inactivates complement (Pillemer *et al.*, 1953). The inactivation of components by plasmin is very similar to those inactivated by antigen-antibody aggregates and in both cases may be due to activation of C′1 proesterase (Lepow and Pillemer, 1955; Lepow *et al.*, 1956).

5. The removal of calcium and magnesium ions prevents the haemolytic activity of complement and a very potent reagent for removing these ions is now available in the form of E.D.T.A. (ethylenediaminetetracetic acid).

6. Storage. The activity of whole complement rapidly deteriorates when sera are kept at 37° C. Whole serum may lose all its complementary activity if kept at 37° C. overnight, or for one week at room temperature. The complement titre would be reduced approximately by half by being maintained at 4° C. for one week. If serum is frozen solid at −35° C. complement is well preserved and will maintain full activity for two weeks, after which its titre diminishes but is preserved at reduced titre for many months (Seifter *et al.*, 1943).

carried out in tubes, 3×½ inch, and ten tubes are set up and numbered The serum to be titrated is diluted 1 in 15 in barbital buffer and an increasing quantity run into each tube, and the total volume is brought up to 0 5 ml. with buffer; 1 ml. of sensitized sheep cells is then added. These are incubated at 37° C. for 30 minutes with regular shaking.

Tube number:	1	2	3	4	5	6	7	8	9	10
Barbital buffer (ml.) ..	0 45	0 4	0·35	0·3	0·25	0 2	0·15	0·1	0 05	0
Human serum diluted 1 in 15 (ml.)	0 05	0·1	0·15	0 2	0·25	0·3	0·35	0·4	0·45	0·5
Sensitized cells (ml.) ..	1·0	1·0	1·0	1·0	1·0	1·0	1·0	1·0	1·0	1·0

Control tests should include 1 ml. of sensitized cells mixed with 0·5 ml. 1 in 15 inactivated test serum.

At the end of the incubation the tubes are removed from the water bath and centrifuged at 1,000 r.p.m. for 5 minutes. The degree of haemolysis is compared with a series of haemoglobin standards made up at 10 per cent intervals and ranging from 10–100 per cent lysis.

Haemolysis %:	100	90	80	70	60	50	40	30	20	10
Water, ml.	0·5*	0 6*	0·7*	0 8*	0·9	1·0	1·1	1·2	1·3	1·4
Sensitized cell suspension, ml.	1·0	0·9	0 8	0 7	0·6	0 5	0·4	0 3	0·2	0·1

* A knife-point of saponin should be added.

The comparison is a visual one to the nearest 5 per cent.

Fifty per cent haemolysis is taken as the end point, but 100 per cent may be used in addition if desired.

A unit of complement is the smallest amount of the test serum that will result in 50 per cent haemolysis under the conditions of the test as described. Thus if Tube 3 shows 40 per cent and Tube 4, 60 per cent lysis, 0·175 ml. of 1 in 15 serum will give 50 per cent. lysis.

$$\text{Units} = \frac{1}{0{\cdot}175} \times 15 = 85 \text{ units/ml.}$$

Normal fresh sera have a range of 60–100 units/ml. and a median value of 80 units/ml.

Inactivation of Complement

There are many ways in which the titre of complement in human serum can be reduced or by which complement may be destroyed; among these are:

1. Heating.
2. Change in pH.
3. Absorption by antigen-antibody aggregates.
4. Action of plasmin.
5. Removal of Ca++ and Mg++.
6. Storage.

1. The normal way of inactivating complement is by heating at 56° C. for 30 minutes. The thermo-labile components, C'1 and C'2 are destroyed, leaving C'3 and C'4 in reduced titre. Heating at 50° C. for approximately 40 minutes may also render complement inactive; C'2 is destroyed (Ecker *et al.*, 1943).

2. The effect of pH change on complement has been studied by Seifter *et al.* (1943). They added various amounts of acid and alkali to human sera and allowed them to stand at room temperature for 1 hour. Haemolytic activity of whole serum was completely destroyed at pH 4·2 and pH 10. It seems likely that complement is completely active between pH 7 and pH 8·4.

3. Treatment of complement with antigen-antibody aggregates results in complete inactivation of the second and fourth components, C'2 and C'4, and partial inactivation of the first component, C'1, whereas there is little or no inactivation of C'3. We have used egg albumin/rabbit anti-egg albumin precipitate for decomplementing sera. It is interesting to note that Weigle and Maurer (1957) have shown that although such antigen-antibody precipitates may be completely saturated with complement, they still remove haemolytic activity from fresh serum.

4. Plasmin prepared by the addition of streptokinase to normal serum inactivates complement (Pillemer *et al.*, 1953). The inactivation of components by plasmin is very similar to those inactivated by antigen-antibody aggregates and in both cases may be due to activation of C'1 proesterase (Lepow and Pillemer, 1955; Lepow *et al.*, 1956).

5. The removal of calcium and magnesium ions prevents the haemolytic activity of complement and a very potent reagent for removing these ions is now available in the form of E.D.T.A. (ethylene-diamine-tetracetic acid).

6. Storage. The activity of whole complement rapidly deteriorates when sera are kept at 37° C. Whole serum may lose all its complementary activity if kept at 37° C. overnight, or for one week at room temperature. The complement titre would be reduced approximately by half by being maintained at 4° C. for one week. If serum is frozen solid at −35° C. complement is well preserved and will maintain full activity for two weeks, after which its titre diminishes but is preserved at reduced titre for many months (Seifter *et al.*, 1943).

Anticomplementary Sera

Some sera are anticomplementary, even when relatively fresh, and others become so after storage; some may be strongly anticomplementary. Anticomplementary activity can be estimated by adding dilutions of the anticomplementary serum to dilutions of serum containing known quantities of complement. During storage of sera, even when stored frozen solid, anticomplementary activity may develop. Contamination of a sample or the presence of soaps may result in anticomplementary activity. Davis *et al.* (1944) have shown that human γ globulin is anticomplementary. They say that heating at 56° C. for half an hour decreases the anticomplementary activity of purified Wassermann antibody. Olhagen (1945) believed that the anticomplementary effect was due to a kind of denaturation of the serum globulin. Sera that are anticomplementary may have a high globulin content, e.g. those from cases of multiple myelomatosis.

The anticomplementary activity which develops in sera on storage may make it difficult to use them as blood grouping sera if the detection of antibody is dependent on fresh serum factors.

From what has been said previously it is obvious that if C′1 esterase is active in the serum due to activation of plasminogen, or for other reasons, the serum might well possess anticomplementary activity. It might be an advantage to heat old stored sera at 56° C. for 45 minutes before adding fresh serum and prior to blood grouping tests.

Absorption of Sera

If it is desired to absorb anti-A and anti-B antibodies from a serum with the minimum reduction of complement, the rapid method described on p. 25 should be used up to Step 8. Absorption should not be undertaken at 37° C. but only at 4° C. If anti-A and anti-B antibodies are present in the serum in high titre, even using the rapid method at 4° C., it will be difficult to avoid some fixation of complement and reduction of its titre. For example, in one experiment such a serum was absorbed as suggested and its titre fell from 85 units per ml. before absorption to 60 units per ml. after absorption. If such sera are absorbed at a higher temperature using slower techniques, a great reduction in complement titre will occur. It is desirable, therefore, to avoid the need to absorb such antisera as anti-Le^a and anti-Jk^a. If it is necessary to carry out absorption at 37° C., e.g. to remove anti-D which may be present with anti-Jk^a in the serum, absorption should be undertaken with group O cells at 37° C. and the anti-A and anti-B removed separately as above. An alternative method to try would be to inactivate such a serum and carry out the necessary absorption and add the fresh serum later, but this is less satisfactory. Nevertheless, some reduction in complement titre is inevitable and with relatively common

antibodies, such as anti-Le^a and anti-Le^b, it is of great value for these to be found in the sera of persons of group AB. Red cells which have been preserved in the frozen state and restored to saline suspension sometimes exhibit anticomplementary activity.

Tests for Haemolysins

Detection

Anti-A, anti-B, anti-Le^a, anti-Le^b, anti-Tj^a, anti-Vel, and occasionally samples of anti-P, may possess haemolytic activity. Anti-Le^a and anti-Le^b haemolysins are more readily detected if papainized test cells are used (p. 295). The test is carried out in 2×⅜ inch tubes and one volume, 0·3 ml. of fresh serum, mixed with one volume of a 3 per cent erythrocyte suspension in barbital buffer. The mixture is incubated in a water bath for one hour at 37° C. with intermittent shaking; the tubes are then spun and the supernate inspected for haemolysis. If this volume of serum is not available a smaller volume can be used together with an equal volume of cells in the detection test. A control test of inactivated serum, together with the cell suspension, is included and forms a standard of reference.

This test will enable the presence or absence of haemolysis to be detected. If the percentage lysis is required the results should be compared with a series of haemoglobin standards. Comparison may be difficult as the proportion of serum in the test is high.

It is difficult to detect haemolysins in old stored sera which lack complement and in sera that possess anticomplementary properties. A volume of fresh serum may be added to a volume of stored serum, together with two volumes of cell suspension, and the test attempted. In the case [illegible]
and Le^b su[illegible]
also be fre[illegible]
such sera are not easy to find.

Titration of Anti-A and Anti-B Haemolysins

The test is performed in 2×⅜ inch tubes. The serum to be tested is used undiluted and diluted 1 in 2, 1 in 4, 1 in 8, etc. in barbital buffer. One volume, 0·3 *ml.* of each dilution is placed in the tubes. A_1 or B test cells are freshly collected in citrate (Chapter II) and washed three times. A 2 per cent suspension is made in a mixture consisting of three volumes of specially prepared or selected fresh serum (*vide infra*) and one volume of barbital buffer. 0·3 ml. is added to each tube. The tubes are placed in a water bath for one hour at 37° C. and shaken frequently during this time to dislodge the agglutinated cells; the tubes are then centrifuged. Complete haemolysis is taken as the end point and is judged by the fact that no cells are precipitated by centrifugation.

A series of colour standards can be prepared and the percentage haemolysis estimated by visual comparison but the presence of excess serum necessary to provide complement for the test makes this comparison difficult and the former method is normally employed. Control tests should consist of one volume of the cell suspension in serum together with one volume of inactivated test serum.

In this test the haemolysin is diluted in barbital buffer and the cells suspended in a diluted fresh serum. The serum can be obtained from two sources:

1. Sera from group AB persons who are non-secretors, as ascertained by saliva and serum tests;
2. Sera from persons of group O, who are selected for weak anti-A and anti-B saline agglutinins only, which can readily be removed by absorption.

The danger of using (1) above, is that the sera may contain traces of A or B specific substance and iso-haemolysins are readily inactivated by these. On the other hand, the danger of using (2) is that absorption of anti-A and anti-B agglutinins will result in a marked reduction of complement titre. In this case, the reduction of complement titre will be least if the rapid absorption method described on p. 25 is used. For comparative purposes, there is not much to choose between a carefully selected serum from a group AB non-secretor person and an absorbed group O serum. Whichever of these two it is decided to use, the serum should be titrated for complement beforehand. Table 20 illustrates a titration and for the purpose of clarity the percentage haemolysis has been estimated. The results with the barbital buffer diluent indicate the very considerable fixation of complement which occurs during this titration.

TABLE 20. TITRATION OF ANTI-A HAEMOLYSIN IN VARIOUS DILUENTS

Dilution of test serum	*% Haemolysis. Test cells suspended in·*			
	Barbital buffer	*AB secretor serum*	*AB non-secretor serum*	*Absorbed Grp. O serum*
1 in 1	90	100	100	100
1 in 2	80	90	100	100
1 in 4	30	80	100	90
1 in 8	0	50	80	80
1 in 16	0	20	30	30
1 in 32	0	0	0	0

Role of Complement in the Antiglobulin Test

Dacie (1950), described a cold incomplete antibody and Crawford, Cutbush and Mollison (1953) showed that it had anti-H specificity (p. 126). This antibody united with red cells only in the presence of fresh serum and at low temperature. On p. 61 we have described the two kinds of antiglobulin reagent and cells sensitized with this antibody react only with anti-non-γ globulin reagent.

Mollison and Cutbush (1955) described anti-Le^a and anti-P antibodies which could be detected by the antiglobulin test only if the patient's fresh serum was used, and remarked that the reactions of these antibodies are similar in some respects to those of incomplete cold antibodies and the incomplete antibody found in cold type acquired haemolytic anaemia described by Dacie (1954). Stratton (1955) stressed the importance of freshly collected samples for optimum results in detecting Lewis antibodies.

At this time Cutbush, Crawford and Mollison (1955) were of the opinion that some antibodies such as incomplete anti-Le^a appear to be α or β globulins. Vaughan and Waller (1956) considered that anti-Lewis antibody and one example of anti-Kidd appeared to be non-γ globulin. Stratton (1956) described an example of anti-Jk^a which like anti-Le^a showed deterioration on storage and an activity which was decreased following inactivation but which was enhanced by the addition of fresh normal serum.

Dacie, Crookston and Christenson (1957) considered that potentially haemolytic cold antibodies sensitized erythrocytes to agglutination by antiglobulin serum. They also stated that "cold antibodies probably do not exist in a demonstrably incomplete form and that the agglutination by antiglobulin serum of erythrocytes exposed to these antibodies is due to an interaction between the antiglobulin serum and sub-haemolytic amounts of adsorbed complement rather than between the antiglobulin serum and antibody. The adsorbed complement protein reacts with antiglobulin serum to which various protein fractions have been added as if the adsorbed protein consisted of β and α_2 globulin." They showed that the presence of three components of complement, C'1, C'2 and C'4 was sufficient to cause agglutination by antiglobulin serum, whereas all four were required for lysis. Christenson, Dacie and Croucher (1957) showed that cold antibodies from a case presenting the "cold haemagglutinin syndrome" fell in the γ_1 fraction.

Vaughan and Waller (1957) showed that if Lewis positive cells were sensitized with decomplemented anti-Lewis serum, washed and incubated with fresh serum, there was a considerable increase in Coombs titre as though complement had been taken up.

Stratton and Ryman (1958) confirmed that Le(a+) cells sensitized with inactivated anti-Le^a serum when washed and treated with fresh

serum would give a positive antiglobulin test, but not if mixed with inactivated serum. Occasionally, positive results were obtained with inactivated serum depending on the manner of decomplementation of the original antibody serum. It was also shown that inactivated anti-Le^b and anti-Jk^a (three examples, one kindly provided by Dr. A. Matson), in addition to anti-Le^a (several examples), would sensitize cells at 37° C. but a positive antiglobulin test could not be obtained until the sensitized cells had been further treated with fresh serum and washed. Dilute fresh serum, up to 1 in 500, would enable some sensitized cells to be detected by the antiglobulin test. Thus the union of antigen and antibody can occur in the absence of whole complement activity, but the antiglobulin test is negative (anti-non-γ reagent) but becomes positive if these sensitized cells are subjected to fresh serum. In the case of Le(a +) sensitized cells giving a negative antiglobulin test and a positive one when further subjected to fresh serum any of the treatments of the fresh serum described on p 53 designed to render complement inactive prevented the occurrence of a positive antiglobulin test; i.e. destruction of C′1, C′2 or C′4 but not C′3.

Cold incomplete anti-H is an exception and fresh serum is needed during sensitization of the cells, and indeed with some other antibodies better results occur under these conditions.

From a practical point of view, therefore, in the antiglobulin test for the detection of these antibodies, the concentration of complement should be as high as possible and the test cells should be suspended in suitable fresh serum and the appropriate antiglobulin reagent used.

References

Christenson, W. N., Dacie, J. V., and Croucher, B. E. E. (1957). Electrophoretic studies on sera containing high titre cold haemagglutinins; Identification of the antibody as the cause of an abnormal γ_1 peak. *Brit. J. Haemat.*, **3**, 262.

Crawford, H., Cutbush, Marie, and Mollison, P. L. (1953). Specificity of incomplete cold antibodies in human serum. *Lancet*, **i**, 566.

Cutbush, Marie, Crawford, H., and Mollison, P. L. (1955). Observations on anti-human globulin sera. *Brit. J. Haemat.*, **1**, 410.

Dacie, J. V. (1950). Occurrence in normal human sera of incomplete forms of autoantibodies. *Nature*, **166**, 36.

Dacie, J. V. (1954). *The Haemolytic Anaemias.* London: J. and A. Churchill Ltd.

Dacie, J. V., Crookston, J. H., and Christenson, W. N. (1957). Incomplete cold antibodies. The role of complement in sensitization to antiglobulin serum by potentially haemolytic antibodies. *Brit. J. Haemat.*, **3**, 77.

Davis, D. B., Kabat, E. H., Harris, A., and Moore, D. H. (1944). The anticomplementary activity of serum γ globulin. *J. Immunol.*, **49**, 223.

Ecker, E. E., Pillemer, L., and Seifter, S. (1943). Immunochemical studies of human serum. I. Human complement and its components. *J. Immunol.*, 47, 181.

Kabat, E. A., and Mayer, M. M. (1948). *Experimental Immunochemistry.* Springfield, Illinois: Chas. C. Thomas.

Lepow, I. H., and Pillemer, L. (1955). Studies on the mechanism of inactivation of human complement by plasmin and by antigen-antibody aggregates. II. Demonstration of two distinct reaction stages in complement fixation. *J. Immunol.*, 75, 63.

Lepow, I. H., Ratnoff, O.D., and Pillemer, L. (1956). Elution of an esterase from antigen-antibody treated with human complement. *Proc. Soc. Exp. Biol. Med.*, 92, 111.

Mollison, P. L., and Cutbush, Marie (1955). Use of isotape labelled red cells to demonstrate incompatibility in vivo. *Lancet.* i, 1290.

Olhagen, B. (1945). Studies on thermo-stable anticomplementary human sera. *Acta Med. Scand.*, Suppl. 162.

Osborn, T. W. B. (1937). *Complement or Alexin.* Oxford University Press.

Pillemer, L. (1943). Recent advances in the chemistry of complement. *Chem. Rev.*, 33, 1.

Pillemer, L., Ratnoff, O. D., Blum, L., and Lepow, I. H. (1953). The inactivation of complement and its components by plasmin. *J. Exp. Med.*, 97, 573.

Pillemer, L., Blum, L., Lepow, I. H., Wurz, L., and Todd, E. N. (1956). The properdin system and immunity. III Zymosan assay of properdin. *J. Exp. Med.*, 103, 1.

Seifter, S., Pillemer, L., and Ecker, E. E. (1943). Immunochemical studies of human serum. 2. In vitro studies on the stability of human complement and its components. *J. Immunol.*, 47, 195.

Stratton, F. (1955). Lewis antibodies. *Lancet*, ii, 143.

Stratton, F. (1956). Value of fresh serum in the detection and use of anti-Jk^a antibody. *Vox Sang.*, 1, 160.

Stratton, F., and Ryman, Mary (1958). To be published.

Vaughan, J. H., and Waller, M. V. (1956). Immunochemical nature of anti-red cell antibodies. *Proc. of the 6th Cong. Intern. Soc. Blood Trans.*, 196.

Vaughan, J. H., and Waller, M. V. (1957). Immunologic features of erythrocyte sensitization. II. The nature of blood group antibodies. *Blood*, 12, 29.

Weigle, W. O., and Maurer, P. H. (1957). Behaviour of complement in antigen-antibody complement precipitates. *J. Immunol.*, 79, 319.

CHAPTER IV

THE ANTIGLOBULIN TEST

THE antiglobulin test was first described in 1945 by Coombs, Mourant and Race, for use in the detection of incomplete Rh antibodies. It is also known as the Coombs test. It is the most versatile and valuable of all tests for the detection of blood group antigen-antibody reactions and is widely used.

Principle of the Test

Cells suspended in saline and exposed to the action of an incomplete blood group antibody are not agglutinated but absorb the antibody on their surface and an antigen-antibody reaction takes place. Blood group antibodies are globulins and consequently the cells become coated with human globulin. If these cells are then washed free from serum and mixed with an anti-human serum or antiglobulin reagent prepared in some other animal, agglutination of the sensitized red cells will occur, indicating that originally there had been an antigen-antibody reaction although no agglutination had been evident using saline suspensions of the cells. It has been pointed out previously that the difference between antibody globulins and normal globulins is a very subtle one that cannot be detected except by immunological means. Thus a general antiglobulin serum will agglutinate most red cells coated with antibody globulin.

The test is of two kinds:

1. The Direct Test.
2. The Indirect Test.

There is no essential difference, in principle, between these two tests.

The Direct Test, was evolved following the work of Coombs, Mourant and Race (1946), who showed that cord cells in cases of haemolytic disease of the newborn were coated with antibody which was demonstrated by the antiglobulin reagent. It is a test which shows that human globulin has united with red cells in vivo.

The Indirect Test, on the other hand, is a test in which erythrocytes are mixed with antibodies in vitro and, if an antigen antibody reaction takes place, globulin is absorbed on to the red cells. Following washing of the mixture a positive antiglobulin test can be obtained. If the erythrocyte antigen is unknown it can be identified with a known antiserum and vice versa.

Anti-γ-, and Anti-non-γ-globulin Reagents

Coombs and Mourant (1947) showed originally that the component

of the antiglobulin serum responsible for the reactions was an anti-γ-globulin and, so far as cells sensitized with Rh antibodies are concerned, this is still thought to be the case. Cells sensitized by antibodies of certain other specificities are agglutinated by other components present in the antiglobulin reagent.

Dacie (1951), Crawford and Mollison (1951), and Renton (1952), all showed that antiglobulin reagents could be absorbed with one type of sensitized cell without abolishing the activity against certain other types, and Renton (1952) prepared two fractions, Fraction I, active against Rh sensitized cells, and Fraction II, active against cells sensitized by cold incomplete anti-H. The addition of a suitable amount of human γ globulin to the reagent is another means of preparing Fraction II.

Unfortunately, preparations of human γ globulin are often referred to as "Cohn's Fraction II", or simply as "Fraction II", when prepared from human serum by Cohn's alcohol precipitation method, so that the terms "Fraction I" and "Fraction II" give rise to confusion when they refer to components of antiglobulin reagents and the terms "anti-γ-globulin" and "anti-non-γ-globulin" are preferable. The anti-γ-globulin fraction is the fraction that reacts most strongly with Rh sensitized cells and is present in high dilution. The anti-non-γ-globulin fraction is that which is still active in the antiglobulin serum after the addition of γ globulin; it is present in lower concentrations in the average serum and is active only against certain varieties of sensitized cells, such as those sensitized by cold incomplete anti-H, anti-Jk^a, most anti-Le^a and anti-Le^b, and occasional examples of other antibodies as well as certain cells coated in vivo, mainly in pathological states. It is possible that it reacts more strongly with $\alpha\,\beta$ globulins and that the antibodies detected by its action are detected because of fresh serum factors absorbed on to the antibody coated erythrocyte.

We fe[illegible]

[illegible] acting as described above or to components present in native antiglobulin serum.

The reactions of sensitized cells with reagents of the two types are shown in Tables 21 and 22, and it is valuable to have both types of reagent available. Firstly, because antibodies can be detected using a reagent containing the appropriate component, and secondly, because the use of separate reagents—the one diluted and the other with added γ globulin—often provides a valuable clue as to the identity of an unknown antibody; and thirdly, because the use of one or other specific component might be valuable when using a particular antibody (example anti-Jk^a as a typing reagent where it was present in serum mixed with other antibodies, for example weak anti-D).

The reactions of the two components with agglutinins can only be investigated by absorbing antiglobulin reagents with washed aggluti-

nated cells and in the case of group A cells agglutinated by anti-A the results are very variable, depending on the individual anti-A serum. Some anti-A sera remove the anti-γ component, some the anti-non-γ, some both, and some neither, and examples of this are shown in Table 23. Furthermore, the previous treatment of the anti-A serum affects the results and Table 24 shows a serum which would absorb the anti-γ-globulin component only after it had been heated.

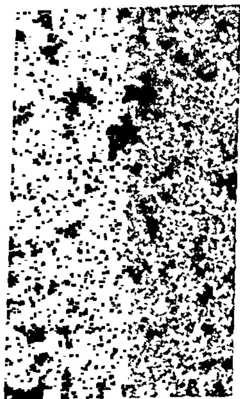
The agglutination produced by the two types of reagent has a somewhat different appearance. That caused by the anti-γ-globulin presents a uniform appearance with little variation in the size of the agglutinates (Plate V*a*). The anti-non-γ-globulin reagent, on the other hand, tends to give a more irregular appearance with agglutinates of varying size (Plate V*b*) and the reaction with the sensitized cells is often slower to develop on the slide than with an anti-γ-globulin reagent.

TABLE 21. INDIRECT COOMBS TEST USING THE TWO MAIN COMPONENTS

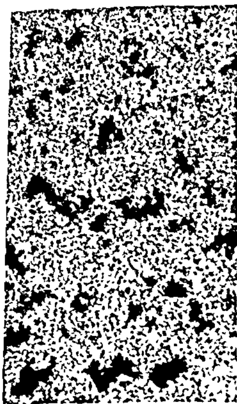
Reagent	*Cells sensitized with*									
	Anti-A	*Anti-Rh*	*Anti-S*	*Anti-K*	*Anti-Fy^a*	*Anti-Le^a*	*Anti-Le^b*	*Anti-Jk$_a$*	*Anti-P*	*Cold Anti-H*
Anti-γ-globulin	— or +	+	+	+	+	— or rarely +	— or rarely +	— a few +	—	—
Anti-non-γ-globulin (γ-globulin added)	+	—	—	— or few +	— or +	+	+	+	+	+

TABLE 22. DIRECT COOMBS TEST USING VARIOUS REAGENTS

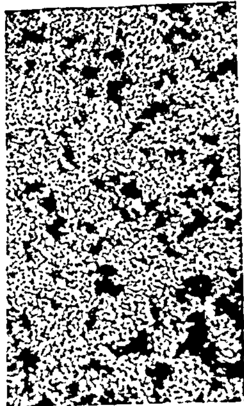
Cells from case of	*Antiglobulin reagent*		*Routine reagent*
	Anti-γ-globulin	*Anti-non-γ-globulin (γ globulin added)*	
Acquired haemolytic anaemia (warm type) ..	+++++	—	+++++
Acquired haemolytic anaemia (cold type) ..	—	++++	+++
Acquired haemolytic anaemia	++++	+++	++++
Acquired haemolytic anaemia (in remission)	+++++	—	+++++
Cord cells, in haemolytic disease of the newborn	+++++	—	+++++



a Coombs test with Rh sensitized cells. The agglutination presents a uniform appearance See p 62. ×220



b. Coombs test with incomplete anti-B sensitized cells. The agglutination presents an irregular appearance. See p. 62. ×220



c. Agglutination of washed cells by colloidal silica. The appearance closely resembles a true positive Coombs test (cf. *a* and *b*) See p. 73. ×220

PLATE V. Coombs test agglutinations.

TABLE 23. REACTIONS OF THE TWO COMPONENTS OF AN ANTIGLOBULIN REAGENT INVESTIGATED BY ABSORPTION WITH WASHED AGGLUTINATED A_1 CELLS

I		*II*										*III*						
Antiglobulin reagent absorbed with		*Test cells sensitized with incomplete anti-D*										*Test cells sensitized with cold incomplete anti-H*						*Component of antiglobulin reagent removed by absorption*
		Dilutions of antiglobulin reagent treated as indicated in column I																
	Anti-A serum No.	4	8	16	32	64	128	256	512	1,000	2,000	4	8	16	32	64	128	
A_1 cells sensitized with different anti-A sera	1	++++	++++	++++	++++	+++	++	+	−	−	−	−	−	−	−	−	−	Predominantly anti-non-γ-globulin
	2	w	−	−	−	−	−	−	−	−	−	++++	++++	+++	+	w	−	Anti-γ-globulin
	3	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	Both
	4	++	+	−	−	−	−	−	−	−	−	++	+	w	−	−	−	Both partly
	5	++++	++++	+++	+++	++	+	w	−	−	−	++++	+++	++	+	w	−	Trace of anti-γ only
Absorbed with unsensitized A_1 cells		++++	++++	++++	++++	+++	+++	++	+	−	−	++++	+++	++	+	w	−	Control
Unabsorbed antiglobulin reagent		++++	++++	++++	++++	+++	+++	+++	++	w	−	++++	++++	+++	++	+	−	Control

II Represents an estimation of the anti-γ-globulin component.

III Represents an estimation of the anti-non-γ-globulin component.

TABLE 24 EFFECT OF HEAT ON ABILITY OF AN ANTI-A SERUM TO REMOVE THE ANTI-γ-GLOBULIN COMPONENT OF A COOMBS REAGENT WHEN THIS WAS ABSORBED WITH A_1 CELLS AGGLUTINATED BY THE ANTI-A SERUM

Treatment of anti-A serum used to sensitize group A_1 absorbing cells	*Reactions of absorbed Coombs reagent with Rh sensitized cells*
Unheated	++++
Heated at 55° C. for 35 min.	++++
,, 56° C. ,,	+++
,, 60° C. ,,	++
,, 65° C. ,,	−

Zoning with Antiglobulin Reagents

High titre antiglobulin reagents often show prozones, and it is necessary to be careful that one does not use a reagent at a dilution falling within the prozone or a false negative result may occur (Table 25). The prozone is always more marked with weakly sensitized cells than with strongly sensitized ones and this is probably due to factors similar to those which cause anti-D sera to show more zoning with D^u than with D cells (p. 172). The prozone is not solely dependent on the sensitized cells, however, and the same sensitized cells may give different lengths of prozone with different antiglobulin reagents of the same titre. This suggests that antiglobulin reagents may contain an incomplete antiglobulin as well as an agglutinating antiglobulin, and this is probably of importance in the detection of weakly sensitized cells (Renton and Hancock, 1958). D^u cells have a weak D antigen and are consequently inagglutinable by anti-D sera containing a high proportion of incomplete antibody and it would seem that weakly sensitized cells might behave similarly with antiglobulin reagents containing incomplete components.

Preparation of the Reagent

The reagent is prepared by the injection of human serum or human serum fractions, parenterally, into animals.

Whole human serum from group O persons is used to avoid secretor substances. We have not found it any advantage to select sera for the absence of Lewis substances. The sera should be rapidly spun before use to precipitate "ghost" cells. Fresh human serum is very satisfactory, but serum which has been heated to 56° C. for 30 minutes can be employed. Serum which has been heated to 65° C. for half an hour will not produce a satisfactory antiglobulin reagent.

We have used both γ globulin and α β globulin as inocula; the former

produces an excellent reagent containing both components, but the latter does not produce a satisfactory reagent of either variety.

Many animals have been used in which to prepare antiglobulin reagents. They should be capable of producing potent reagents which should stand up to the tests described later. It should readily be possible to absorb the serum to leave the specific activity relatively undiminished.

Our experience has been that rabbits are the animals most likely to meet these requirements; the sheep has the advantage that a much larger quantity of blood can be obtained from it. Assuming the animals are to be kept alive and bled from time to time, 30 ml. is the average quantity that is removed from the rabbit, whereas 400–500 ml. can be obtained from the sheep. Through the courtesy of our colleagues we have tried the effect of injecting a number of animals. Monkeys will produce an antiglobulin reagent, but we have not injected a sufficient number to tell whether they are able to produce very good ones. Mice do not appear to be able to produce satisfactory reagents at all. Horses produce a reagent, but the one we tried had a high titre non-specific activity against normal red cells. Reagents can also be produced in hens. For the production of small quantities of reagent there is no doubt that rabbits are the best laboratory animals.

INOCULATION SCHEDULE

The inoculum consists of whole fresh serum from group O persons (0·5 ml.–1 ml. for a rabbit), suspended in Freund's adjuvant (1947). One-third of this is given *intramuscularly* and the *remainder subcutaneously*, preferably at more than one site. The rabbit is then left for a period of 4 to 6 weeks, after which a second injection of 0·5 ml. of whole fresh serum without adjuvant is given intramuscularly. The animal is bled 12 days after this second injection. The serum is now examined and if it is considered that the animal is likely to produce a good antiglobulin reagent it is kept for future inoculation.

Ideally, the animals should now be left for a period of 8 weeks, or longer, before further inoculations are given, but at least they should be left for several weeks, following which an inoculation of 0·5 to 1 ml. of whole serum is given intramuscularly and after 12 days the animals bled again. In many cases the selected animals, following this interval and third inoculation, will give satisfactory antiglobulin reagents. If the reagent is not entirely satisfactory, it is often advantageous to give an inoculation of 50 mgm. γ globulin subcutaneously and bleed and re-test in 10 days. If the response has been poor, further inoculations should be given and the serum retested.

Sheep have a similar inoculation schedule, but larger doses are given and the time interval between the first and second dose should be 8 weeks or longer.

Innumerable methods have been suggested for the production of

antiglobulin reagents in animals. Both whole serum and γ globulin have been used successfully as inocula, either on their own or succeeding one another and with or without the addition of adjuvant, or serum has been used as alum precipitated serum. Inoculation schedules vary, as does the ability of the animals to produce good reagents. We feel that the following points are noteworthy:

1. If whole serum is used, the initial dose should be given in some manner which will delay its absorption. We prefer an emulsion of the serum with Freund's adjuvant. The amount of serum injected into the rabbit should not be excessive; we employ 0·5–1 ml.
2. Those animals that are likely to produce good reagents should be selected by testing their sera at an early stage.
3. Following this, an interval of time should be left before further doses of serum or γ globulin are given. The advantage of this interval technique is that a high titre antiglobulin reagent can be prepared using a minimal number of injections of serum from which, in turn, it is less difficult to prepare a specifically active antiglobulin serum.

ABSORPTION

Serum should be inactivated (56° C. for 10 minutes). Titrations are then carried out to determine its activity with normal red cells. Certain rabbit sera may appear to be inactive against normal red cells by a slide technique, but agglutination will occur if sedimentation tests in tubes are employed. On the other hand, sheep sera will often show a higher titre, such as 8–32, against normal red cells using a slide test.

Absorption should be carried out with cells of blood group O (several examples), group A_1 and group B. The red cells are collected in A.C.D. solution as described (p. 19). Great care should be taken during their collection to see that they are thoroughly mixed with the anticoagulant. This is to avoid the occurrence of any flecks of fibrin or small clots which will be spun down with the red cells and may tend to neutralize the antiglobulin reagent. Thorough washing of the cells is essential. They are first packed down and all the plasma and buffy-coat removed. 10–20 ml. of packed cells are pipetted into a large bottle with 500 ml. of normal saline and washed once. The cells are then removed to smaller containers and each 2–3 ml. of them is washed with 20 ml. of saline several times. It is difficult to determine when all the serum has been washed free from the cells and various methods have been tried, but none is entirely satisfactory. Stratton and Jones (1955) showed that repeated absorption of an antiglobulin reagent with normal red cells, even when these had been thoroughly washed, would reduce the titre of such a reagent against Rh sensitized cells, whereas absorption of the reagent with trypsin-treated cells would not cause this reduction.

Papainized red cells act similarly to trypsin treated cells. Absorption with papainized cells alone may fail to remove all the antibody activity against normal red cells.

The procedure is to absorb one volume of reagent for 30 minutes with one volume of washed packed papainated cells of groups O, A and B, mixed together, at room temperature. The serum will then usually have little activity against the absorbing cells and it is further absorbed at 4° C. and 37° C. with ½–1 volume of mixed normal cells as may be required. Tests to ensure complete absorption should include saline agglutination tube tests at 16° C. and 37° C. for 1½ hours. The reagent will then be suitable for use on slides at room temperature.

It is necessary to use a number of normal red cells for these tests, as occasional cell samples may be found which react positively with an antiglobulin reagent. If it is proposed to carry out the test by a technique other than that described (for example, a technique employing tubes and a centrifugation method), tests for complete absorption of the Coombs reagent should be made using this technique. Similarly, if diluents other than saline are to be used in the Coombs test, e.g. albumin, then the reagent should be tested using albumin suspended test cells.

Dilution alone to produce a specific serum is unsatisfactory, since the chance of an occasional normal red cell giving a positive result is increased. A serum diluted alone gave weak positive results with three out of fifty normal cells. Rabbit sera will normally give some reaction with papainized red cells and with infected red cells. The titre will vary from serum to serum, but if it is proposed to use an antiglobulin reagent using the enzyme cell Coombs technique described by Unger (1951), then it is essential to absorb with enzyme treated red cells.

PREPARATION OF ANTI-NON-γ-GLOBULIN REAGENT

The principle is to eliminate the anti-γ-globulin activity of a concentrated, and suitably selected, antiglobulin reagent by the addition of human γ globulin, and the amount added should be such as to remove all activity against cells strongly sensitized by anti-D whilst causing as little reduction of the activity against cells sensitized by incomplete anti-H as possible. The amount required should be determined by suitable titrations, but is not usually critical, and with many reagents the following is successful.

To 10 volumes of the absorbed undiluted antiglobulin reagent is added 1 volume of a 1 in 100 solution of γ globulin. The mixture is allowed to stand at 4° C. for 2–3 hours and rapidly centrifuged to remove the precipitate and then diluted to the appropriate dilution. Care should be taken to remove all precipitate.

The γ globulin used should be the purest obtainable; impure preparations may reduce the efficacy of the reagent. If no suitable pre-

paration of γ globulin is available a concentrated antiglobulin reagent without addition of γ globulin can be used. The anti-non-γ-globulin reagent whose reactions are described in this book is one to which γ globulin has been added, but equally good results would be obtained with a suitable concentrated reagent without added γ globulin. The only difference is that the latter type of reagent is likely to have anti-γ-globulin activity also, even though it is used at a dilution far removed from the optimal dilution for anti-γ-globulin activity, so that the diagnostic value of the former type of reagent, which is often useful in the identification of antibodies, is lost. The term "anti-non-γ-globulin" in general, therefore, includes both types of reagent.

Testing

The standardization of an antiglobulin reagent is hard to define and such guidance as is given here is intended to apply to reagents which it is proposed to use for routine purposes in a general laboratory. The results of titrating an absorbed rabbit antiglobulin serum with various sensitized cells are shown in Table 25. The titre of the serum will depend upon the degree to which the cells are sensitized and this will vary with the technique of sensitization and with the particular antibody concerned.

This is a form of titration that enables one to assess the suitability of a reagent and the dilution at which it should be used. This Table shows:

1. The failure of the serum to agglutinate normal cells.
2. That this particular serum should be used in several dilutions, by preference as an anti-γ reagent at 1 in 100 for a single dilution, but better still used at three dilutions: 1 in 80, 1 in 160, and 1 in 320; as an anti-non-γ reagent at 1 in 12.
3. The activity of the reagent is abolished by the addition of γ globulin against cells sensitized with certain antisera, but not against those sensitized with others.
4. That the titre with Rh sensitized cells may be much higher than for other kinds of sensitized cells, and may be misleading as the sole indicator of the value of a reagent.
5. The occurrence of zoning is observed when weakly sensitized cells are used and this occurs when anti-Rh sera are used as well as, to a lesser extent, with anti-Le^a and similar antibodies. This means that with weakly sensitized cells an optimum reaction occurs over a more restricted dilution range, as the vertical line in Table 25 shows.

This antiglobulin serum has been particularly selected for titration [illegible] considerable degree of zoning and serves to illustrate [illegible]

[illegible]

because its [illegible]

TABLE 25. TITRATION OF RABBIT ANTIGLOBULIN SERUM AGAINST CELLS SENSITIZED WITH VARIOUS ANTISERA. VERTICAL MARK INDICATES OPTIMUM RANGE WHEN USED WITH THOSE PARTICULAR SENSITIZED CELLS. CONTROL TESTS NEGATIVE

Titre	*Anti-D strong*	*Anti-D weak*	*Anti-K*	*Anti-Fy^a weak*	*Cells from active warm acquired H. anaemia*	*Cord cells from haemo. disease of newborn*	*Anti-Le^a*	*Anti-Le^a weak*	*Anti-Jk^a*	*Cold incompl. anti-H*	*Normal cells.* Gp. O i	ii	iii	Gp. A_1	Gp. B
2	+++	–	+	–	+	+	+++++	++	+++	+++++	–	–	–	–	–
4	++++	–	+	w	++++	+++	**+++++**	**+++**	**+++++**	**+++++**	–	–	–	–	–
8	+++++	–	++	+	**+++++**	+++++	**+++++**	**+++**	**+++++**	**+++++**	–	–	–	–	–
16 ..	**+++++**	w	**+++++**	++	**+++++**	**+++++**	**+++++**	**+++**	**+++++**	**+++++**	–	–	–	–	–
32 ..	**+++++**	++	**+++++**	+++	**+++++**	**+++++**	++++	++	++++	++++	–	–	–	–	–
64 ..	**+++++**	++	**+++++**	**+++**	**+++++**	**+++++**	++	+	++++	++	–	–	–	–	–
128 .	**+++++**	**+++**	**+++++**	**++++**	**+++++**	**+++++**	+	w	+++	+	–	–	–	–	–
256 ..	**+++++**	**+++**	++++	**++**	**+++++**	**+++++**	w	–	++	w					
512 ..	**+++++**	++	+++	–	**+++++**	**+++++**	–	–	+	–					
1,024 ..	+++++	+	++	–	+++++	+++++	–		–	–					
2,048 ..	+++++	w	+		+++	+++			–						
4,096 ..	+++	–	–		++	++									
8,192 ..	++	–	–		+	+									
16,000 ..	+				w	–									
32,000 ..	–				–	–									
64,000 ..	–				–										
Saline ..	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Effect on titration results of addition of γ globulin	All Neg.	All Neg.	All Neg	All Neg.	All Neg	All Neg.	Unaffected	Unaffected	Reduction of titre	Unaffected					

TABLE 26. PROZONE DURING TITRATION OF ANTIGLOBULIN REAGENT AGAINST JK(A+) SENSITIZED CELLS

Cells weakly sensitized with	*Dilutions of anti-non-γ-globulin reagent*									
	1	2	4	8	16	32	64	128	256	*Control*
Anti-Jk*	−	+	++	++++	+++	+++	+	−	−	−

weakly sensitized cells, may be greater, although the strength of an individual reaction may be less, but not invariably so.

Not all reagents react in the manner illustrated in Table 25. Some may show little activity against Le(a+) sensitized cells, or have a high titre against cells strongly sensitized with anti-Rh antibody but be inactive against weakly sensitized ones. Such reagents may be unsuitable for use. Not all the ideal features may be present in one reagent and it may be possible to use a particular reagent as an anti-γ reagent but not as an anti-non-γ reagent, and vice versa. The anti-non-γ reagent to which γ globulin has been added should give negative results with Rh sensitized cells and strongly positive results with cold sensitized cells at the dilution at which it is proposed to use the reagent. The reasons for using a more concentrated reagent to which γ globulin has been added have been previously stated, but the essential thing is to use antiglobulin reagents in such dilutions that all kinds and degrees of sensitized cells will be detected.

Every antiglobulin reagent needs to be used for a while in the laboratory, firstly to see that the supposed optimum dilutions are, in fact, the best, and secondly, to test it with many varieties of sensitized cells to ensure that it is generally useful.

STORAGE

The reagent is best stored in the undiluted but absorbed state and diluted as may be required, say at weekly intervals. The diluted antiglobulin reagent should have a stabilizer added to it because dilute protein tends to become denatured and the titre of the reagent falls on storage. Bovine albumin has been used as an additive, but in some circumstances we have found that this causes the reagent to give false positive results and haemoglobin in a final concentration of 1 per cent is recommended. Both the concentrated reagent and the dilute reagent should be stored frozen solid at −20° C. when not in use. This helps to preserve their sterility although antiseptics, such as sodium azide, 0 1 per cent, may be added.

Technique of the Test

DIRECT COOMBS TEST. The blood is collected as described on p. 18 and two drops are placed in a 3×½ inch tube. These are then

washed four times in normal saline, filling the tube on each occasion. The test is performed on microscope slides at room temperature. The slide is divided into two with a grease pencil and 1 volume (0 03 ml.) of a 5 per cent suspension of washed red cells placed on each side. On the left-hand side an equal volume of antiglobulin reagent is added and on the right-hand side a volume of saline. These are then mixed, with an applicator stick or plastic rod, into a small circle about ½ inch in diameter. A stop watch is then started. The slide is gently tilted and the result recorded as observed by the naked eye at 2, 4 and 6 minutes, and finally the test read under the low power of the microscope and recorded. Similarly the control test is examined. Typical results would be as follows:

	2 min.	4 min.	6 min.	*Microscopic examination*	*Control test*
Positive result ..	++	++++	+++++	+++++	−
Negative result	−	−	−	−	−

At the conclusion of a negative test 1 volume of a 5 per cent suspension of thoroughly washed Rh sensitized cells (not strongly sensitized, p. 181) is added to each side of the slide and rocked for 6 minutes. The side on which the antiglobulin reagent has been placed should give a positive result and the control test a negative one. This ensures that the antiglobulin reagent is active and that the cells being tested were adequately washed. If a concentrated reagent is being used in the test, or an anti-non-γ-globulin reagent, cells sensitized with cold incomplete anti-H antibody should be used as control cells in place of the Rh sensitized cells.

INDIRECT COOMBS TEST. The indirect test is used to detect unknown antibodies using standard cells or alternatively to detect red cell antigens using known typing sera. The reagents are mixed in 3×½ inch tubes; 2 volumes of serum together with 1 volume (0 1 ml.) of a 10 per cent suspension of red cells. The mixture is incubated at the appropriate temperature, usually 37° C. for 45 minutes. The serum is then removed from the sedimented cells, and the cells washed four times in saline, removing the supernatant as completely as possible each time. The test then proceeds as described under "Direct Coombs Test" above.

Tests should ensure that the antiglobulin reagent is acting specifically and it should preferably be used in several dilutions. The saline control test should be negative.

ENZYME CELL COOMBS TEST. Unger (1951) described this test in which trypsinized red cells are substituted for normal cells in the indirect test. If careful attention is paid to the erythrocyte concentra-

tion the correct dilution of antiglobulin reagent and other details of the test, most antibodies will be detected using normal cells. In cases where the test is weakly positive, the substitution of enzyme treated cells for normal cells may do no more than increase the strength of the test cell and control cell tests almost equally. Moreover, the test is liable to give false positive results and controls are essential.

In certain cases, such as the detection of anti-Jk[a] antibodies, this method may succeed where others fail.

False Positive and False Negative Results

These are dealt with generally in Chapter V, but there are a number of special difficulties that can affect the antiglobulin test and these are referred to below.

False Positive Results

1. Chemical Contamination. In agglutination tests there is usually a high concentration of serum present. This is an efficient buffer and is also able to combine with and neutralize traces of many chemical substances. Chemical contamination has, therefore, to be fairly marked before a false positive result will occur. The washed, serum-free cells of the antiglobulin test are much less able to resist the action of traces of extraneous chemical substances, dirty glassware, etc.

2. Poor Quality Saline. Saline containing dust or other particles is a cause of difficulty. In washing the cells during the test, particles present in the saline collect at the bottom of the tube during each centrifugation so that all the particles from all the washings become concentrated into one or two drops of cell suspension and may result in difficulty in interpreting the test.

3. Colloidal Silica (Stratton and Renton, 1955; Renton and Hancock, 1957). Certain preparations of colloidal silica have an extremely potent agglutinating effect on red cells and are able to agglutinate them even in dilutions as high as one part per million. Other preparations exert a blocking effect and prevent agglutination by the agglutinating preparation, though cells treated with such preparations may be agglutinated by certain dilutions of serum and they sometimes agglutinate spontaneously on washing. In other words, it is only when

[illegible]

saline and other solutions used in serological work and give rise to difficulties in the antiglobulin test. The solutions of trisodium citrate used in the glycerol citrate method of preserving red cells in a frozen state (p. 20) are very liable to contamination by colloidal silica if

sterilized by autoclaving in glass containers. Trisodium citrate attacks glass at the temperature of the autoclave and colloidal silica may be formed which can give rise to a false positive Coombs test using frozen cells. Colloidal silica is readily neutralized by serum proteins and accordingly does not cause difficulty in agglutination tests with a high concentration of serum present. The amount of serum present in a dilute antiglobulin reagent is often insufficient to neutralize the colloidal silica.

Another way in which silica causes difficulty is in the mixing of the anti-globulin reagent with the sensitized cells on a microscope slide. If the reagents are stirred vigorously with a glass rod there is often sufficient abrasive action between the two glass surfaces to cause agglutination either through the formation of colloidal silica or through the action of fresh surfaces of finely powdered glass. This phenomenon is easy to demonstrate by placing one drop of a 5 per cent suspension of thoroughly washed red cells on a microscope slide and stirring vigorously with a glass rod. If the slide is gently rocked, as in the antiglobulin test, agglutination will readily be observed. Tap water often contains fine particles of sand which will increase the abrasive action if left on the microscope slides. For these reasons it is recommended in the antiglobulin test that the cells and serum should be mixed with a plastic rod or applicator stick.

It should be understood that the phenomenon is only of occasional occurrence and it is capricious in nature, i.e. it will occur perhaps only in a single test because the conditions for the action of colloidal silica just happen to be right. The appearance of the agglutination due to colloidal silica often simulates a true positive Coombs test very closely (Plate Vc), and may develop in a similar manner when the cells are rocked on the slide. One should, however, be suspicious of cells which stick markedly to the glass surfaces of the tube [illegible] during the washing of the cells.

If a tube breaks in the centrifuge during the performance of the test it is inadvisable to salvage the cells from the bottom half of the tube, since these may contain particles of broken glass and we have known colloidal silica to be formed under these conditions causing false positive results.

4. **Inadequately Absorbed Antiglobulin Reagent.** If the reagent is inadequately absorbed and reacts positively with normal cells it will, of course, give false positive results in the test. Care, however, has usually been taken to absorb the reagent satisfactorily at the outset but, like all immune sera, there is a tendency for non-specific antibodies to return to the serum during storage.

The serum should, of course, be sterile and reference has already been

made to the fact that normal rabbit serum may react with papainized red cells. Care should, therefore, be taken to avoid this difficulty in using the enzyme cell Coombs test.

5. Use of Cells with a Positive Direct Coombs Test. In the direct test, of course, the object is to determine whether cells are coated with antibody, but in the indirect test using standard cells, or testing cells with standard sera, it is presumed that the cells which are used are not already coated with antibody. This difficulty might occur for two reasons:

1. The use of clotted cells which are coated with cold incomplete anti-H antibody (Dacie, 1950); or,
2. The use of cells from normal persons which give a positive direct Coombs test.

The characteristics of cold incomplete anti-H antibody are described in Chapter VI. If cells are collected as clotted blood or defibrinated blood, and stored at refrigerator temperature (4° C.), then they may become coated and give a positive direct Coombs test, especially when tested with the more concentrated antiglobulin reagent, i.e. anti-non-γ-globulin reagent. When samples are collected in the form of clotted blood and stored for a short period of time at 4° C., e.g. overnight, the number of samples that give a positive result is small.

A number of blood donors have been found whose red cells have a positive direct Coombs test. These were detected because incompatibilities were observed in the cross matching test. Stratton, Stone and Tovey (1958) have observed eight such cases.

Case 2

Donor, aged 44 years, married. One full-term normal pregnancy eleven years ago; no abortions, no blood transfusion. She had given twenty-one blood donations during the previous eight years but had never had any serious illness. A full haematological examination revealed no abnormality. Her blood groups were: O,R_1R_1, MNs, P-neg. Lu(a —), Le(a+b—). Other blood group tests could not be performed because of the positive direct Coombs test. Wassermann reaction and Berger Kahn tests were negative. The donor has been observed for three years during which time her direct Coombs test has remained positive. Results of testing her cells are shown in Table 27.

Case 3

Blood donor, aged 55. Had an appendicectomy in 1926 and pneumonia in 1932, otherwise his health had been good. He never received any blood transfusions. He had given ten donations of blood. His blood groups were A_1, R_1R_1, MNS, P-negative, Lu(a—),

Le(a−b−). Wassermann reaction and Berger Kahn tests were negative. The direct Coombs test has now been known to be positive for four years. The results of tests are shown in Table 27. In this case, unlike the previous case, there is a history of pneumonia which is known sometimes to precede acquired haemolytic anaemia. This may be the cause of the erythrocyte change in this particular blood donor, although he is, at the present time, in excellent health.

TABLE 27. RESULTS OF DIRECT COOMBS TESTS ON ERYTHROCYTES OF CASES 2 AND 3

Cells	*Antiglobulin reagents*	
	Anti-γ-globulin	*Anti-non-γ-globulin*
Case 2	++++	—
Case 3	+	+++
Cold sensitized cells	—	++++
Rh sensitized cells	+++++	—

6. CO-AGGLUTINATION. Co-agglutination (Coombs *et al.*, 1955), is a phenomenon in which agglutination occurs when unsensitized cells, human serum and antiglobulin reagent are mixed together. Presumably the cells become enmeshed in a precipitate formed by the antiglobulin reagent and human serum. The phenomenon causes similar false positive results to the immune adherence phenomenon of Nelson (1953). Here, red cells adhere to an immune precipitate. If partially washed red cells are used and mixed with an antiglobulin reagent a precipitate will occur to which the red cells may become adherent. This may be mistaken for a true positive result, but can be distinguished by the appearance of the precipitate.

7. SYPHILIS. A weakly positive direct antiglobulin test is sometimes found in cases of syphilis. This may cause confusion when the cord cells of infants suffering from congenital syphilis are tested using the direct Coombs test. In these cases the clinical picture may occasionally resemble haemolytic disease of the newborn. We have occasionally observed weak positive results in adults having a positive reaction for syphilis. It is useful to remember here that persons suffering from acquired haemolytic anaemia may have a false positive serum test for syphilis.

8. THE USE OF SERA CONTAINING SALINE AGGLUTININS. If a serum containing a saline agglutinin is tested using the indirect test it sometimes happens that the agglutinates are broken up during the washing process but reform when the cells are rocked on a slide with antiglobulin reagent. An agglutination will occur in the saline control

but may be different in strength from that which occurs when the antiglobulin reagent is used. This is not strictly speaking a false positive result. Sturgeon (1954) has described an augmentation agglutination reaction, which depends on the difference between the two results.

9. INFECTED TEST CELLS. Antiglobulin sera if used in a concentrated form may agglutinate infected red cells. Normal rabbit serum will sometimes agglutinate infected cells to a titre of 8.

10. UNKNOWN. Occasionally apparently false positive results occur for unknown reasons. The indirect Coombs test using the patient's own cells and serum will usually give the same strength of reaction as when using the patient's serum and other test cells.

If all the precautions listed in this chapter are observed, these false positive results are usually only weakly positive, are of uncommon occurrence, and do not cause difficulties of interpretation.

FALSE NEGATIVE RESULTS

1. INACTIVE REAGENT. Menolasino and Davidsohn (1954) described a method of heating rabbit antiglobulin reagents to render them specific. Hunter and Thomas (1957) found that this adversely affected the anti-non-γ fraction. We find the effect to be variable but often to result in deterioration of the anti-non-γ-globulin fraction and to make the serum less able to agglutinate weakly sensitized cells.

The presence of human serum at any part of the test will inactivate the reagent. Pipettes used for the antiglobulin reagents are kept separately; an in-dwelling pipette in the reagent itself is desirable, but if this is not available special pipettes should be kept for this purpose. If an anti-non-γ-globulin reagent containing added human γ globulin is to be used as a test reagent, a special pipette should also be kept for this, since, of course, γ globulin will inactivate an anti-γ reagent. The precaution of adding Rh sensitized cells sensitized to a suitable degree or cold sensitized cells at the conclusion of the test is a precaution to ensure that the antiglobulin reagent itself has been active during the performance of the test.

Partial inactivation of Coombs reagent may occur and affect the ability of such a reagent to detect weakly sensitized cells (Table 28). This error may be difficult to detect and the Rh sensitized cells added at the conclusion of the slide test should not therefore be too strongly sensitized. Partial inactivation can also occur during absorption of the reagent.

Inadequate washing of the cell suspension is a very common cause of contamination by serum. Many diluted Coombs reagents would be completely or partially inactivated by an equal volume of a 1 in 5,000 dilution of human serum, so that a high standard of washing is needed. The considerations involved may be illustrated by a simple example.

Suppose that there are three or four drops of serum in the tube and that 3 ml. of saline is added. This will dilute the serum to about 1 in 20. If as much as three or four drops of this mixture remain in the tube when the supernatant is removed after centrifugation, the dilution after the second washing will be about 1 in 400 and after the third washing, 1 in 8,000. Such cells would be insufficiently washed. The procedure described on p. 71 should be adhered to; if larger volumes are used, a larger tube will be required to ensure adequate washing. Neglect of these considerations is a common cause of false negative results in the direct Coombs test.

TABLE 28. PARTIAL NEUTRALIZATION OF ANTIGLOBULIN SERUM

Cells sensitized with	*1 vol. antiglobulin serum (1 in 50) + 1 vol. saline*	*1 vol. antiglobulin serum (1 in 50) + 1 vol. serum (1 in 5,000)*	*Saline*	*Serum (1 in 5,000)*
Strong anti-D ..	+++++	+++++	—	—
Weak anti-D ..	++++	—	—	—
Weak anti-Fy^a ..	++++	—	—	—

2. PROZONE. Some antiglobulin reagents show prozone when titrated with sensitized cells [illegible]

3. DOSE OF TEST CELLS IN INDIRECT TEST. If the proportion of test cells to serum in the indirect test is too great then a false negative result may occur. Where known weak antibodies are involved, it may be desirable to reduce the proportion of red cells to serum below that [illegible] ortion of cells [illegible] cells must be sufficient for the performance of the test. The 10 per cent cell suspension recommended is satisfactory for ordinary purposes.

4. TECHNICAL ERRORS. Technical errors may result in false negative results. The antiglobulin test as undertaken on slides at room temperature is essentially a developing test and the agglutinates are friable. If the slide is shaken violently or dropped on the bench, agglutination may not occur or may disappear. In order to effect maximum agglutination within the time of the test the slide should be carefully and gently rocked.

5. THE VALUE OF FRESH SERUM. In Chapter III it has been shown that certain antibodies, such as anti-Le^a, anti-Le^b, anti-Jk^a, although they may unite with the antigen, do not give a positive antiglobulin test

using anti-non-γ-globulin reagent unless fresh serum factors, probably complement, have been absorbed on to the aggregate. In the case of cold incomplete anti-H, fresh serum needs to be present during the period of sensitization. In practice it is desirable to have fresh serum present during the process of sensitization for these antigen-antibody reactions to be detected by the antiglobulin test. If fresh serum is not present, a positive result will not be obtained even though antigen-antibody union has occurred.

The Value of the Test

The antiglobulin test is a valuable test and is used under the following circumstances:

1. THE DIRECT TEST

(*a*) For testing cord blood samples in suspected cases of haemolytic disease of the newborn when it is due to Rh, ABO, or other incompatibility between mother and child.

(*b*) In cases of acquired haemolytic anaemia when the cells of the patient give a positive direct Coombs test with either one or other of the components of the antiglobulin reagent.

(*c*) As an indication that incompatible transfusions have occurred. Here the picture will show small clumps of cells in a field of unagglutinated cells, depending on the length of time which has elapsed since the incompatible transfusion was given; this is an indication that the cells are transfused cells and that they are coated with the patient's antibody (Plate XV*b*).

2. THE INDIRECT TEST

(*a*) For the detection of atypical antibodies in human sera:
   i. During antenatal testing;
   ii. In the cross matching test.
   iii. For the purpose of detecting atypical antibodies in any serum (for example, following transfusion reactions).

(*b*) For blood grouping purposes such as:
   i. The detection of Rh antigen D^u, using a strong incomplete anti-D antibody.
   ii. Typing of red cells, e.g. with anti-Fy^a, anti-Jk^a.
   iii. For the identification of human blood; this is employing a neutralization test and can be used on dried blood stains, etc., for forensic purposes.

Various alternative methods have been described of carrying out a direct Test to detect cells coated with antibody in vivo. Witebsky, for example, has described a test carried out on slides using the cells mixed with whole serum, or with albumin. Nevertheless, the antiglobulin test is very widely used for the purpose and is of considerable

diagnostic value in haemolytic disease of the newborn. Similarly, in cases of acquired haemolytic anaemia it is of diagnostic value, although persons who are perfectly healthy can give positive direct Coombs tests as can those who have suffered from acquired haemolytic anaemia and whose clinical condition is in a state of remission. The antiglobulin test is one of the tests of choice for cross matching (Chapter X). In antenatal testing it is also a valuable test, although for detecting anti-D the papain cell test is better. For the detection of certain antigen-antibody reactions, antiglobulin tests are absolutely necessary (e.g. Jk^a-anti-Jk^a). Although some other antisera, such as anti-Fy^a, have been known to act as saline agglutinins, the typing of cells for this group and the detection of anti-Fy^a usually necessitates the use of the antiglobulin test. For many purposes, therefore, it is a test for which there is no satisfactory substitute.

Most Rh antibodies are detected by this test. Occasional ones during the early months of pregnancy are positive only using enzyme treated cells, but in many cases these react in the antiglobulin test shortly afterwards. Anti-Kell, anti-Fy^a, anti-Le^a, anti-Le^b and anti-Jk^a can all be detected using this test and the appropriate antiglobulin reagent.

In short, therefore, the antiglobulin test is a valuable test for which in some circumstances, there is no alternative and which is preferentially used in a large number of blood grouping procedures.

In the indirect test, if various practical points, such as the dose of red cells, antiglobulin dilution, etc., are adhered to, when the test is used, the worker will find few occasions on which he will need to resort to the enzyme cell antiglobulin test.

References

Coombs, R. R. A., Mourant, A. E., and Race, R. R. (1945). A new test for the detection of weak and "incomplete" Rh agglutinins. *Brit. J. Exp. Path.*, 26, 255.

Coombs, R. R. A., and Mourant, A. E. (1947). On certain properties of [illegible]

[illegible]

5[illegible], 1[illegible].

Coombs, R. R. A., Mourant, A. E., and Race, R. R. (1946). In-vivo iso-sensitization of red cells in babies with haemolytic disease. *Lancet*, i, 264.

Coombs, R. R. A., Saison, Ruth, and Joysey, Valerie (1955). A circumstance in which the phenomenon of co-agglutination could complicate the antiglobulin sensitization tests by simulating agglutination. *Brit. J. Exp. Path.*, 36, 179.

Crawford, H., and Mollison, P. L. (1951). Demonstration of multiple antibodies in antiglobulin sera. *Lancet*, ii, 955.

Dacie, J. V. (1950). Occurrences in normal human sera of "incomplete" forms of cold autoantibodies. *Nature*, 166, 36.

Dacie, J. V. (1951). Differences in the behaviour of sensitized red cells to agglutination by antiglobulin sera. *Lancet*, ii, 954.

Freund, J. (1947). Some aspects of active immunization. *Ann. Rev. Microbiol.*, **1**, 291.

Hunter, D. A., and Thomas, A. R. (1957). The effects of heating anti-human globulin sera. *J. Clin. Path.*, **10**, 245.

Menolasino, N. J., and Davidsohn, I. (1954). A simplified method for preparation of antihuman globulin (Coombs) serum. *Am. J. Clin. Path.*, **24**, 1205.

Nelson, R. A. (1953). The immune adherence phenomenon. An immunologically specific reaction between micro-organisms and erythrocytes leading to enhanced phagocytosis. *Science*, **118**, 733.

Renton, P. H. (1952). Separation of Coombs reagent into two fractions. *Nature*, **169**, 329.

Renton, P. H., and Hancock, Jeanne A. (1957). Antibody-like effects of colloidal silica. *Vox Sang.*, **2**, 117.

Renton, P. H., and Hancock, Jeanne A. (1958). Rh antibodies and the prozone phenomenon. *J. Clin. Path.*, **11**, 49.

Stratton, F., and Jones, A. R. (1955). The reactions between normal human red cells and antiglobulin (Coombs) serum. *J. Immunol.*, **75**, 423.

Stratton, F., and Renton, P. H. (1955). Effect of crystalloid solutions prepared in glass bottles on human red cells. *Nature*, **175**, 727.

Stratton, F., Stone, B., and Tovey, G. H. (1958). Unpublished observations.

Sturgeon, P. (1954). The nature of "complete" Rh antisera as revealed by antiglobulin augmentation. *J. Immunol.*, **73**, 212.

Unger, L. J. (1951). A method for detecting Rh- antibodies in extremely low titer. *J. Lab. and Clin. Med.*, **37**, 825.

CHAPTER V

THE CAUSES OF FALSE RESULTS IN AGGLUTINATION TESTS

One need only engage in blood grouping work for a short time before encountering false positive or negative results in some of the tests; indeed a knowledge of such errors and the ways of recognizing and avoiding them is essential to all who undertake this work in however modest a way. It is fortunate that the end result of almost all the techniques used in blood grouping is the presence or absence of agglutination. This means that the possible causes of error are to a large extent the same in all techniques, so that we can consider the causes of false positive and negative agglutination in general terms which will be applicable to all the methods.

Failure of the Human Element

It is the experience of all workers in this field that carelessness and mistakes are a far more frequent cause of difficulty than the purely technical causes of error. For every incompatible transfusion due to erroneous grouping or cross matching there are several due to such things as inadequate indentification of the patient, sticking the compatibility label on the wrong bottle, writing "A" when one means "O", and so forth. Of eleven cases of ABO incompatible transfusion which came to our notice and in which sufficient details were given to determine the cause, only one was due to technical difficulties (infected grouping serum), one was partly due to technical difficulty and partly due to confusion over the patient's name (Case 22, p. 100), and the remaining nine were solely due to failure of the human element.

So difficult is it to avoid mistakes and so serious can be their consequences that it is worth while to examine in detail some cases in which carelessness led to ABO incompatible transfusion.

Case 4

Two patients in the same ward had the same name and the blood cross matched for one was given to the other.

Case 5

The serial number on a bottle of blood chanced to be the same as the serial number on a patient's case notes, which was erroneously thought to indicate that the blood was intended for that patient.

Case 6

The donor's name, which appeared on the bottle, happened to be the same as the name of a patient and an ABO incompatible transfusion resulted.

Following this, we took to covering the donor's name on our bottles of blood, but this precaution did not prevent the following:

CASE 7

A patient was unfortunate enough to have a name which coincided with the name of a town at which a bottle of blood had been collected. An ABO incompatible transfusion resulted.

CASE 8

In a busy hospital, blood for two patients, one group A and one group AB, was cross matched at the same time. All the tests were correctly carried out but the compatibility labels were each stuck on the wrong bottle of blood, and each patient received the blood intended for the other.

Mistakes of this nature are almost inevitable unless the technician carrying out the grouping and cross matching tests is able to work on a reasonable amount of bench space, and in reasonably quiet surroundings and without being continually harried by phone calls and other interruptions.

A type of clerical error which we have seen result in an ABO incompatible transfusion is caused by the habit, which some technicians have, of writing their results on odd scraps of paper and subsequently transferring them to the cross matching card or laboratory record sheet.

The immediate direct recording of results as they are observed is essential to accuracy in laboratory work since the original record is inevitably superior in accuracy to any copy. Copying out results should be reduced as much as possible, and the provision of suitably printed forms and cards on to which the results can be directly written is not only desirable from the point of view of neatness, but is a very good safety factor. Specially designed racks to hold the tubes used in the tests are also very desirable for safety, and these should be labelled and the tubes in them numbered as clearly as possible. Furthermore, the spaces on the forms and cards should, if possible, be arranged in the same order as the holes in the rack in which the tubes for the corresponding tests are to be placed, so that the work can be carried out in an orderly manner. It is also important to develop orderly methods of work, and to work to a set routine. For example, in testing with a number of anti-sera, always keep to a fixed order, e.g. anti-A, anti-B, anti-C, anti-D, anti-E and anti-c. If more than one serum of the same specificity is employed, arrange them in alphabetical order of the names of the sera or numerical order of their identification numbers. If both types of identification are employed, put numbers before names. Tubes should always be placed in racks from left to right and from top to bottom in the same way as one would read a book, and all racks must be marked to show which way round they

should be used. We have often experienced mistakes which were due to inadvertently turning the rack round during the test.

Adherence to such methods and knowledge of the risks of mistakes arising from their neglect would have avoided cases such as the following:

CASE 9

In cross matching blood for two patients, a hospital technician first prepared separate serum and cell suspensions from the specimens of blood provided. These were placed in tubes labelled with the patients' names. Both patients were found to be group AB. Realizing that the occurrence of two AB patients together was unusual, he was very rightly suspicious and retested the grouping, with the same results. Unfortunately he only retested the same cell suspensions and not the original specimens, not realizing that he had, in fact, prepared both tubes of serum and of cell suspension from the same sample. One patient was, in fact, group AB and the other was group O. AB blood was cross matched for both patients without incompatibility being detected and an incompatible transfusion resulted.

Rigid adherence to every detail of a set routine is essential, as shown by another case.

CASE 10

A hospital technician placed blood from two patients in labelled tubes and centrifuged to separate cells and serum. [illegible] t[illegible] b[illegible] name, with the result that each patient received the blood intended for the other.

The correct identification of the patient is by no means as simple a matter as it might appear, and we usually think it necessary to have Christian names, surname, address and date of birth before being reasonably certain. Even these particulars are not certain to be sufficient, however, as was shown by the following case:

CASE 11

Two brothers married girls of the same age and the same Christian name. They all lived in the same house and both wives became pregnant at the same time. We thus had two women, who incidentally had different blood groups, who had the same Christian name, the same surname, the same address, the same year of birth, and almost the same expected date of delivery.

This sort of thing is not uncommon, and it should be remembered that parents often pass on their Christian names to their children, and that even people with the most unusual surnames are likely to have relatives of the same name living in the vicinity. It should not be assumed

that those with unusual names are in any less danger of incompatible transfusion than those with common names.

A special case of this sort of difficulty arises in the case of newborn infants whose blood is very commonly confused with that of the mother, either through carelessness or through the ignorance of junior nurses who sometimes seem to think that the maternal end of the umbilical cord contains maternal blood, or else that the blood which drips from the raw surface of the placenta is foetal in origin. If unexpected results are obtained in tests of cord blood, it is always worth while considering whether one is, in fact, testing maternal blood, and in the following case considerable difficulty arose from the faulty collection of a cord blood specimen:

CASE 12

Mrs. H., group Orr, had no Rh antibodies in her serum, and gave birth to twins, one of which became jaundiced. A sample of blood was received labelled "Cord blood of Twin II". On testing the cells from this sample with anti-A and also with anti-D, cell mixtures were seen with both agglutinates and free cells present. At first it was thought that this was an example of a chimera, and in order to confirm this hypothesis a fresh sample was obtained from Twin II and also a sample from Twin I. These samples showed that Twin I was group OR_1r and Twin II was group AR_1r. No evidence of cell mixtures was seen on testing these samples. This disproved the idea of a chimera since not only were the original findings not confirmed but also Twin I was Rh positive, so that it could not have been the source of the Rh negative cells present in the original sample from Twin II. Returning to the original sample, we removed the A cells by agglutination with anti-A serum and found that the remaining group O cells gave no agglutination with anti-D sera. This sample was, therefore, a mixture of group A Rh positive and group O Rh negative blood, and it was concluded that it was probably a mixture of blood from Twin II and the mother. Further tests showed that Twin II was suffering from haemolytic disease due to anti-A.

It may perhaps seem to the reader that mistakes such as those described above are of such an obvious nature as hardly to require mention. However, we make no apology for their inclusion since it has been our experience that such errors are a far more potent and frequent cause of difficulty than the purely technical ones, and it is a matter of extraordinary difficulty to get people to avoid them. One difficulty is that blood grouping work requires an exceptionally high [illegible]

to mix a drop of a cell suspension with a drop of anti-D serum and to

read the result, yet one mistake in setting up, reading or recording the result may mean that a woman or girl will perhaps be immunized to the D antigen. Or it may mean that a patient will have an incompatible transfusion.

Such are the grave consequences of error.

Technical Causes of False Results

There are a great many things that can go wrong with the tests employed in blood grouping work. Some, such as autoagglutination, bacterial and chemical contamination, rouleaux, cell mixtures and the various difficulties due to clotting, are of everyday occurrence. Most of the other causes mentioned in this chapter are less frequent but they do occur from tim [illegible]
the occurrence of [illegible]
could be said to [illegible]
liable to occur, and it must be explained that the effects described below are not mere theoretical possibilities but are, in almost all cases, difficulties which have actually occurred in our experience and which we have known to cause an erroneous result.

The causes of false results may be divided into causes of false positive and of false negative results, and into factors affecting the erythrocytes, factors affecting the serum and factors affecting the technique of the test. This arrangement leads to a certain amount of duplication, but it is of value since it provides a scheme for the elucidation of difficulties. If one is confronted with an unexpected result in testing a serum with a sample of erythrocytes in any test, the first question to ask is whether the same result is obtained on testing the same cells with other sera and on testing the same serum with other cells. In many cases this will enable one to suspect that something is wrong with the cells, serum or technique, as the case may be. This should go a long way towards tracking down the cause of difficulty. It should perhaps be mentioned that when factors affecting the serum are referred to this may apply to any serum used in the test. This may be a standard typing serum, a patient's serum in a cross matching test, or perhaps an antiglobulin serum or the serum present in a serum albumin test. All may be affected by many of the factors mentioned.

FALSE POSITIVE RESULTS DUE TO FACTORS AFFECTING THE ERYTHROCYTES

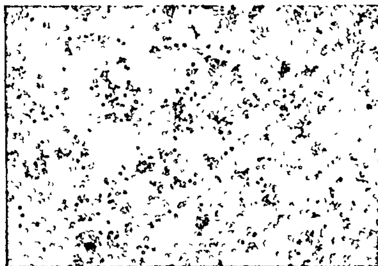
1. AUTOAGGLUTINATION. If a patient's serum contains an autoagglutinin and if the sample of blood has been cooled before use, it will frequently happen that the erythrocytes are agglutinated. If cells which are already agglutinated are tested with typing sera, they will probably remain agglutinated during the performance of the test, and a false positive result will ensue. Autoagglutinins unite with red cells at

a low temperature and the reaction is reversible, so that the antibody can be eluted off the cells by raising the temperature. The thermal amplitude varies from case to case, but the minimum temperature needed for elution is always several degrees higher than the maximum temperature at which the antibody will unite with the cells.

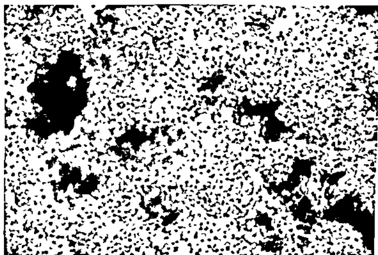
It is essential to start any test with a cell suspension free from agglutination, and it is often useful, particularly with cross matching specimens, to remove a few erythrocytes suspended in their own serum from the specimen and examine microscopically at the outset. In this way, autoagglutination and rouleaux formation, the two commonest causes of difficulty, can be detected at an early stage. If the sample thus examined appears to show agglutination, a few drops of saline should be added and the slide rocked for a couple of minutes. Rouleaux will then usually disperse if weak, or become weaker if strong. Autoagglutination will also sometimes disperse at this stage and this phenomenon is apt to be mistaken for rouleaux. This is due to the elution of autoagglutinin of low thermal amplitude on dilution of the serum. Usually, however, autoagglutination disperses only slightly on the addition of saline and in this case the slide should be very gently warmed by passing it through a Bunsen burner flame, judging the temperature of the slide on the back of the hand. Autoagglutination will in almost all cases completely disperse at 37° C. by this method and frequently the agglutination will reappear as soon as the slide cools off on the bench. Care and experience are needed in this test, however, since excessive heat will disperse agglutination due to other causes. Also, the dispersal of rouleaux on the addition of saline is a rather slow process, taking perhaps two minutes or longer. It is assisted by rocking the slide. The reappearance of the agglutination which occurs on cooling is often a good indication of the presence of autoagglutination, but here again it is possible to be misled by the fact that rouleaux which have been dispersed by the addition of saline may reappear when the serum again becomes concentrated by evaporation, if the slide is left standing on the bench for a long time.

Having decided that autoagglutination is occurring, the next step is to prepare a cell suspension that is free from agglutination. In some cases this can be done simply by washing in saline at room temperature and this is always worth trying. Other cases will require one or more washes in warm saline at 37° C. and occasionally autoagglutination associated with acquired haemolytic anaemia is not dispersed even at 37° C., and washing at 42° C. or even 44° C. is necessary (see p. 246). Once a free cell suspension has been obtained, no further difficulty should be experienced in testing the cells with any anti-sera desired, but it should not be forgotten that some autoagglutinins contain an incomplete component which is not removed by washing at 37° C. This

PLATE VI.



a Infected cells agglutinated by anti-T. See p 87. ×220



b. Agglutination of polyagglutinable cells by normal serum See p 87 ×220

can cause the cells to have a positive direct Coombs test with Coombs reagents containing anti-non-γ-globulin components.

2. BACTERIAL CONTAMINATION. Samples of blood are always liable to bacterial contamination, either from poor technique in collecting the samples or during subsequent handling, and this may make the cells agglutinable by normal sera. This is the Thomsen-Hübner phenomenon and is due to an antigenic change in the red cell, so that an antigen "T" can be detected in infected cells but not in normal cells. The changed cells then become agglutinable by anti-T agglutinin present in the sera of normal adult individuals.

The agglutination between normal sera and infected erythrocytes can be observed on slides, in tubes or capillaries at room or lower temperature, but not at 37° C. The strength of agglutination varies according to the temperature and method of the test, the degree to which the cells are changed, and the strength of anti-T in the serum. If cell samples are heavily infected and much changed, most sera will agglutinate them strongly by a tube saline agglutination technique, and the anti-T may even have a titre of 8. If, on the contrary, the cells are slightly changed, only a few sera may agglutinate them. In one experiment slightly infected cells were agglutinated by only one of twenty test sera.

Infected red cells if injected into rabbits will immunize the animals and increase the titre of the antibody already present which is active against infected cells. Such a rabbit anti-infected cell serum can be made specific by absorption, so that it will agglutinate infected cells but not normal ones. Such a serum may detect the effect of bacterial action on erythrocytes before human sera will agglutinate them. Different organisms, however, may produce different changes in erythrocytes.

Infected red cells do not give a direct or indirect positive antiglobulin test at 37° C.

The appearance of bacterial agglutination of red cells is often rather different from that seen in agglutination due to blood group antibodies and, with experience, can be recognized or suspected (Plate VI*a*). Infected cell samples may have a purple discoloration, show some haemolysis, or have a foul smell. This is a common cause of difficulty; sniff the blood sample and look for haemolysis if in doubt.

If a sample of whole blood has become infected, the serum in the sample will often fail to agglutinate the cells of the sample, even though these may be agglutinated by a high proportion of other sera.

3. POLYAGGLUTINABILITY. This is a rare condition. It is a condition in which a person's red cells are agglutinated by a variable percentage of normal sera. We have observed ten cases in twelve years. Possibly

an incidence of 1 in 100,000 blood samples. (Eight of these have been published previously. Stratton, 1954; Stratton *et al.*, 1958.) Nevertheless, when it is encountered it can give rise to a most confusing picture.

The person's erythrocytes are agglutinated at low temperature but not generally at 37° C. by sera of compatible ABO group, using saline agglutination techniques on slides or in tubes. The affected cells are not agglutinated by the person's own serum. The proportion of sera giving positive reactions ranges from 9 to 90 per cent. In one case (Stratton *et al.*, 1958) the patient's red cells were agglutinated by some normal sera at 37° C. The indirect Coombs test between the affected cells and agglutinating sera at 37° C. is negative.

Polyagglutinability is a transient phenomenon and a second sample of blood taken after a few weeks' or months' interval usually no longer shows the phenomenon. The condition has occurred in patients suffering from septic abortion, peritonitis, pelvic abscess, haematemesis, septicaemia, etc., and in apparently healthy persons.

It has been suggested that the change in the red cells is due to the activation of the T antigen by bacterial enzymes. Certainly polyagglutinable cells are antigenically different from normal cells, as rabbit immune sera show, and they are agglutinated by specific rabbit anti-infected cell sera. Since the change produced by certain bacterial infections of blood is so similar to polyagglutinability, the latter can only be diagnosed on a sterile sample of blood. The difficulties that arise as a result of this condition are twofold; firstly in ABO grouping, secondly when identifying rare blood group antigens.

CASE 13

A hospital technician grouped a patient's cells as Group A. On cross matching with group A blood, incompatibility was detected. The sample was referred to us and, using a number of anti-A and anti-B sera of different batches, it was found that the group could be read as A, B, AB or O according to which sera were used. The correct group was O. The error would have been avoided if both cells and serum has been tested in determining the ABO group.

CASE 14

Mr. B., aged 75, suffering from haematemesis, required a transfusion, and a sample of blood was sent for cross matching. The result of preliminary ABO grouping is shown in Table 29.

Here was a puzzle—an apparent Group B with anti-B in the serum (X). Grouping was done on slides and it was observed that the patient's serum with A_1 and A_2 cells was pink. The serum was inactivated with the results shown (Y). Further tests with other anti-B typing sera gave the results shown (Z). The patient's cells were polyagglutinable but positive results were given with only 9 per cent of normal sera.

TABLE 29. ABO GROUPING IN CASE 14

	Patient's Blood					
	Cells		*Serum*			
	Anti-A	*Anti-B*	A_1	A_2	*B*	*O*
X*	−	+++	−	−	++	−
Y*	−	+++	+++++	++++	++	−
Z*	−	−	+++++	++++	++	−

* XYZ (see text).

A number of antigens are present on red cells (Table 117, p. 313). Some of the corresponding antibodies are of natural occurrence, but are present in less than 9 per cent of normal sera, which is the lowest percentage of normal sera with which we have seen a polyagglutinable cell react. This is of diagnostic value as is the fact that usually agglutination occurs only at room temperature and the indirect Coombs test is negative. Ultimately diagnosis depends on analysing the effect of immunizing rabbits with the changed cells, and the cross-reactions of the immune serum.

4. CHEMICAL CONTAMINATION. There are various chemical substances which can cause agglutination if accidentally introduced into the specimen. If a hospital reports a number of cases of weak false positive agglutination occurring in a sporadic manner, it may be found that the syringes used for venepuncture are being stored in some antiseptic solution. Alternatively, the washing of the specimen bottles or laboratory glassware may be unsatisfactory. Attention to these matters is usually found to resolve the difficulty. The sense of smell is again useful here, and many a sample with which false positive results have been obtained is found to smell of Lysol, Dettol or alcohol. Some synthetic detergents have a most potent agglutinating action, and their use is not recommended for washing serological glassware. It is desirable to keep the glassware used for serological work entirely separate from that used for other purposes. Neglect of this precaution was the cause of trouble in the following case:

CASE 15

In cross matching blood for a child, agglutination was observed in the saline tests at 37° C. and at 16° C. both with the donor's cells and the patient's own cells. Enquiry showed that the sample of blood from the patient had been collected in a tube found in the bacteriology laboratory, and the sample had a strong smell of phenol. A further sample was obtained in a clean tube and the cross matching was carried out without difficulty.

Some chemical substances can produce specific changes in the reactivity of the red cells. Periodate for example, can cause an antigenic change, and if periodate treated cells are injected into animals a serum can be produced which will agglutinate periodate treated cells but not normal cells (Moskowitz and Treffers, 1950).

Formalin causes a general and apparently non-specific reduction in the agglutinability of the cells (Moskowitz and Carb, 1957).

5. CELL MIXTURES. *It sometimes happens that there are present in a* patient's circulation not only his own cells but also donor's cells remaining from previous transfusions (Plate VII*a*). This can cause either a false positive or a false negative result depending on the circumstances. It can also lead to doubt as to which are the patient's cells and which the donor's, as in the following case.

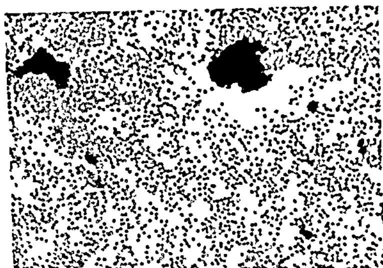
CASE 16

Mr. K., suffering from a very severe haematemesis, was given an emergency transfusion of four bottles of Group O blood. Meanwhile he was grouped as A and five bottles of group A blood were given immediately afterwards. A sample of his blood received by us shortly afterwards had an obvious mixture of agglutinated and free cells when tested with anti-A sera. This mixture was so marked as to make us wonder whether this patient was really group A as stated or whether he was group O with incompatible group A cells still present in his circulation. No anti-A was present in his serum, but this was of no help in determining his group, since anti-A might well be lacking from the serum of a group O patient recently transfused with five bottles of group A blood. It was only by careful consideration of the relative proportion of A and O cells present as compared with the amount of blood given and the estimated extent of the haemorrhage that we were able to convince ourselves that he was in fact group A.

In most cases, however, provided that one is familiar with the transfusion history of the patient, there is no real difficulty, but when one is not familiar with the history difficulty may occur.

Recognition of the fact that two types of cells are present is not always easy. Some unagglutinated cells are always present even with the most powerful reagents, and the condition is only likely to be noticed when one gets the impression that the number of these is increased. This depends on a knowledge of the usual behaviour of the sera concerned. If the condition is suspected it is best to repeat the test using the strongest available sera and to try to form a rough estimate of the relative proportions of the free and agglutinated cells. Where a mixture of Rh positive and Rh negative cells is concerned we have found that a clear-cut picture can best be obtained by means of indirect Coombs tests using incomplete anti-D sera.

PLATE VII.

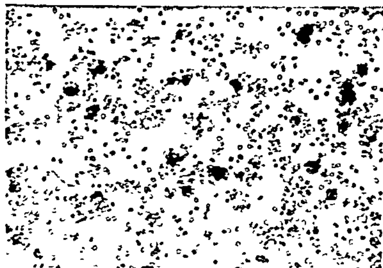


a. Mixed agglutination with anti-B The patient (Group B) had been transfused with six bottles of Group O blood See p. 90. ×220

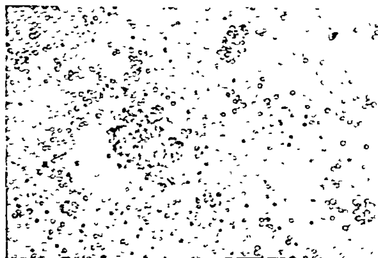


b D positive foetal cells in the circulation of a D negative mother Indirect Coombs test using incomplete anti-D. See p. 91. ×220

PLATE VIII.



a False agglutination due to white cells. Red cells are sticking to the clumps of white cells See p. 92. × 220



b False agglutination due to white cells in a case of myeloid leukaemia. The clumps consist of white cells, the red cells are unagglutinated. See p. 92. × 220

CASE 17

C.P., a case of leukaemia, was group A and was thought to be D positive; he had received seventeen bottles of blood over a period of five months, and his blood was referred to us since there had been no satisfactory rise in the haemoglobin level. We found

was obtained with

r to be an unusual

rect Coombs tests

with the patient's cells sensitized with incomplete anti-D sera definitely showed more free cells than in control tests using normal D positive cells. The direct Coombs test was negative and no abnormal antibodies could be detected in his serum. It was concluded that a mixture of D positive and D negative cells was present in the circulation and the question arose as to whether the D negative cells present were patient's cells or donor's cells. Our donor records showed that sixteen of the bottles he had received had been typed as D positive and one as R'. The latter had been given four months previously. It was, therefore, concluded that he was probably D negative and transfusion with Rh negative blood was advised.

of D positive

using saline

the indirect

serum at any

stage. It would have been interesting to see whether Rh antibody developed when all D positive cells were eliminated. The patient died before this was possible.

A common instance of a cell mixture occurs when babies suffering from haemolytic disease of the newborn have been transfused with Rh negative blood. However, such cases should not cause difficulty.

Cell mixtures in the absence of transfusion occur in the chimera. Cell mixtures also occasionally occur in women at or shortly after delivery, due to the presence of foetal cells in the maternal circulation (Chown, 1954, 1955; Dunsford, 1957).

CASE 18

(Investigated in collaboration with Dr. R. F. Jennison.) Mrs. B., group Orr, no atypical antibodies present, was delivered of an $R_1^u r$ (high grade D^u) child whose

anti-D showed a number of small agglutinates due to the presence of D positive foetal cells. These were particularly clearly shown by treating the mother's cells with incomplete anti-D and carrying out an antiglobulin test (Plate VII (*b*)). The mother's blood had a foetal haemoglobin content of 8 per cent (normal adults less than 2 per cent). D positive cells separated from the maternal blood by a differential agglutination technique yielded a

haemoglobin which was predominantly foetal in character, though the quantity separated was insufficient for quantitative determination. A month after delivery D positive cells could still be detected in the mother's blood, though in fewer numbers than immediately post partum. Two months after delivery no D positive cells could be detected, and the foetal haemoglobin content of her blood was 1 per cent. It was interesting to find that no Rh or other antibodies could be detected in the patient's serum at any time up to six months after delivery.

6. LEUCOCYTES. In the presence of a high white cell count, the leucocytes present may agglutinate, either from the action of citrate or perhaps of anti-leucocyte antibodies, and sometimes erythrocytes stick to the agglutinated masses of leucocytes (Plate VIII*a*). Under low power microscopy it is difficult to see that the agglutinates are composed of leucocytes, though a somewhat "fluffy" appearance of the agglutination is suggestive. Reading naked-eye it is sometimes possible to observe that the clumps are white or grey in colour. In leukaemias with very high white counts the phenomenon can occasionally be quite troublesome (Plate VIII*b*).

7. —D— AND STRONG D. Homozygous —D—/—D—, heterozygous —D— and Strong D cells are occasional causes of false positive results when using saline agglutinating anti-C and anti-E sera which contain incomplete anti-D. See p. 199.

FALSE POSITIVE RESULTS DUE TO FACTORS AFFECTING THE SERUM

1. AUTOAGGLUTININS. The question of preparing a free cell suspension from cells affected by autoagglutination has already been dealt with. The presence of autoagglutinins in the patient's serum can also present difficulty when the serum is tested as part of the determination of the ABO group, for the detection or identification of antibodies or in the cross matching test.

In such cases it often happens that saline or albumin agglutination tests at 37° C. are really negative, but in reading the tests the cell suspensions become cooled whilst on the microscope stage and agglutination occurs. It is advisable, therefore, to read the tests on warm microscope slides and to decide as quickly as possible whether or not agglutination is present. The longer one looks down the microscope in such cases the more marked does the autoagglutination become. Microscope slides can conveniently be warmed by passing them through a Bunsen flame and judging the temperature on the back of the hand. The cold

Autoagglutinins sometimes have an incomplete component (in-

complete anti-H acting as an autoantibody) and this cannot be eluted from the cells at 37° C. In carrying out the Coombs test in such cases, therefore, cells and serum should be warmed to 37° C. as soon as possible after mixing. In severe cases it might even be necessary actually to mix the reagents at 37° C. Furthermore, at the end of the period of incubation the tubes should be rapidly filled with warm saline for the first washing before their contents have had time to cool. The object of these manoeuvres, of course, is to have the serum in contact with the cells at low temperatures as little as possible.

By such means one can, in most cases, satisfy oneself that the tests are negative at 37° C., but occasionally an exceptionally potent autoagglutinin acts so rapidly on even slight cooling that it is necessary to remove it from the serum before any tests can be attempted. This can be accomplished by absorption with the patient's own cells at 4° C. It is often desirable to wash the patient's cells at 37° C. before using them for this purpose, so as to remove the autoagglutinin with which they are coated and thus increase their absorptive power.

The most powerful autoagglutinin we have ever encountered required six absorptions with equal volumes of the patient's own cells before it was sufficiently weakened for further testing to be feasible. We re-used the same cells of the patient in the successive absorptions, washing them at 37° C. between one absorption and another. Autoagglutinins are sometimes associated with an acquired haemolytic anaemia and the absorption process described above is not very desirable in such cases, since specific antibodies to antigens on the patient's cells may also be present and these could be removed by absorption.

Many sera contain an autoagglutinin active at 4° C., so that any tests carried out at this temperature require rigorous controls. For this reason we do not think that tests at 4° C. are very useful save in exceptional cases. Autoagglutinins active only against papainized cells are also sometimes seen and are diagnosed by the reaction with the patient's own papainized cells. Indeed, tests with the patient's [illegible] ntification of antibodies by any [illegible] resemble anti-H in having a [illegible] A or group B cells (Table 30).

People are sometimes confused by the variable degree of agglutination of the patient's own cells, taken directly from the sample, as compared with the degree of agglutination seen when the serum is subsequently tested with other cells. This depends on the thermal history of the sample prior to the separation of cells from serum. If the sample has been kept fairly warm most of the autoagglutinin remains free in the serum, and there will be but little agglutination of the patient's cells in the sample, whilst if the sample has been refrigerated it is likely that the reverse will occur. It is, therefore, best to separate the serum from the cells at a low temperature.

TABLE 30. TITRATION OF AUTOAGGLUTININ AT ROOM TEMPERATURE SHOWING STRONG REACTION WITH GROUP O CELLS (Stratton, 1943)

Serum	ABO group	Titre with test cells				
		A_1	A_2	B	O	Own
Case 1	A_1	2	4	512	16	2
„ 2	A_1	4	8	256	32	4
„ 3	A_1	4	16	256	64	8
„ 4	B	128	128	128	256	64
„ 5	O	64	32	16	16	16

Occasionally, autoagglutinins behave in an unusual fashion, and may appear to belie their name.

CASE 19

The serum of R.H., who was group A_2, Rh negative, agglutinated a selection of standard group O cells at 16° C., but did not agglutinate the patient's own cells. It was at first thought that a specific antibody of high frequency was present, but attempts to identify this failed. The antibody was not active at 37° C. in saline, serum albumin, or by the Coombs test. It was shown not to be anti-Tj^a, anti-k or anti-Le^a+anti-Le^b and seemed unlikely to be anti-O or anti-H, since the patient was A_2 and the antibody reacted equally well with a number of A_1, A_2 and O cells. The patient's direct Coombs test was strongly positive with anti-non-γ-globulin reagents only. Absorption at 4° C. with the patient's own cells which had been previously washed at 42° C. was found to remove the agglutinin. [illegible]
[illegible]
[illegible]
autoantibody with which they were coated.

The antigen or receptor on the red cell surface with which autoagglutinins react appears to be of a rather labile nature, and we have often been misled, using standard cells which had not all been collected on the same day, into thinking that a specific antibody was present. Frozen cells in particular seem liable to give weaker results. In one case, where the sample of the patient's blood was a few days old, and was tested with freshly collected standard cells, it was the patient's own cells that gave unduly weak results. Even using freshly collected test cells, there is some variation in the strength of reaction, and using our panel of standard cells we have come to recognize a characteristic pattern of results which suggests to us that the antibody we are investigating is an autoagglutinin. In the above case an autoagglutinin was mistaken for a specific antibody; the reverse can also occur.

CASE 20

The serum of one patient reacted at 16° C. with a number of cell [illegible] at due to anti-P [illegible] recently received [illegible] his serum with his own cells at 16° C. was due to the presence of P positive donor's cells in his circulation. The fact that only some of the cells present were agglutinating had originally been overlooked.

2. BACTERIAL CONTAMINATION. Bacteria can produce effects on sera which are quite distinct from those they have on cells (Davidsohn and Toharsky, 1940). They produce a panagglutinin, i.e. the infected serum will agglutinate all cell samples, usually most strongly at low temperature. This can be very dangerous and in one case the use of infected anti-A resulted in a grouping error in a group O patient which led to a severe incompatible transfusion.

The panagglutinin is sometimes called anti-h, but it should be noted that this antibody has of course no connection with anti-H of the ABO system. Stratton, Renton and Hancock (1958) described a case of polyagglutinability with a panagglutinin present in the serum which might be anti-h. Both the polyagglutinability of the cells and the panagglutinin in the serum were transient phenomena.

It is not usually practicable in blood grouping work to use a rigidly aseptic technique, but sterile materials and apparatus should be used whenever possible. Furthermore, jars containing tubes and slides contaminated with blood should receive a thorough and regular cleaning lest they become reservoirs of infection. We have not thought it desirable to add antiseptic to the jars, however, since the danger of bacterial contamination would then be replaced by the danger of chemical contamination. It is also most important to keep all sera as cold as possible in order to retard bacterial growth. They should be frozen when not in use, and re-frozen as soon as possible afterwards. Neglect of these precautions will result in the serum giving a "sticky" appearance to the negative results. Later the negatives become weakly positive, and as the infection proceeds it becomes more and more difficult to tell a positive from a negative result. A good serum should give clear-cut negative results; failure to do so may be a danger signal.

Diluted Coombs reagent is particularly liable to bacterial effects. In one experiment a Coombs reagent was deliberately infected with a culture of a paracolon recovered from a reagent found to give false positive results. After only three days at 4° C. the experimentally infected reagent gave strongly positive results with washed non-sensitized cells.

3. CHEMICAL CONTAMINATION. This produces similar effects to

those seen when the serum is bacterially contaminated. The sources of contamination have already been considered (p. 89).

4. ROULEAUX. Rouleaux formation is caused by the presence of large elongated molecules in the serum. Fibrinogen is such a molecule, and so is dextran and polyvinylpyrrolidone. These last two substances cause intense rouleaux formation and if they are to be given to a patient it is most important that a sample of the patient's blood should be taken first, *otherwise considerable difficulty is likely to be encountered in* subsequent grouping and cross matching tests. Intense rouleaux formation also occurs in certain conditions in which abnormal proteins are present in the serum. This regularly happens in multiple myelomatosis and we have also seen it in an undiagnosed case in which an abnormal protein of high molecular weight was present in the serum. Another such case, where a tentative diagnosis of myelocytic leukaemia had been made, had a high globulin-albumin ratio and a high thymol turbidity but no Bence-Jones protein was present. *In both these cases* the serum was so viscous that it was quite difficult to handle with a Pasteur pipette, as it may also be in multiple myelomatosis. In the second case rouleaux even occurred when the serum was diluted 1 in 3 in saline. Less intense rouleaux formation is seen in a variety of pathological states; it is accentuated by slight drying up of the cell suspension on the slide and sometimes with albumin or serum-albumin cell suspensions. Many rouleaux-forming sera lose their rouleaux-forming properties if stored frozen for a few days, a procedure which can sometimes be useful.

Whilst the recognition of rouleaux is sometimes easy, it is sometimes extremely difficult. The classical "pile of coins" appearance is easily *recognized* (Plate IX*a*), *and even if this cannot be seen it is usually* possible to see that the arrangement of the cells resembles a distorted pile of coins (Plate IX*b*). In other words, in rouleaux the cells are predominantly stuck together by their flat surfaces, whilst in agglutination they are stuck together in a much more disorderly manner, any part of one cell being stuck to any part of another. This can best be seen at the edges of the small clumps. The way in which the rouleaux are built up results in three features which assist in the recognition. Firstly, the clumps of rouleaux tend to have a smooth though serpiginous outline; agglutinates have a more irregular outline. Secondly, rouleaux have a more translucent or refractile appearance than agglutinates. Thirdly, the way the clumps move when the slide is tilted is different from the way in which agglutinates move. The clumps of rouleaux tend to move across the field of vision with a rolling motion and give the impression of being rather glutinous and easily distorted. Agglutinates tend to be more rigid. In very strong rouleaux all that can be seen is a massive clump of cells, and it is necessary to break these

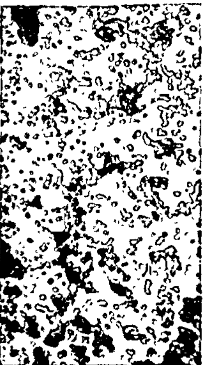
PLATE IX. *Rouleaux.* *See p. 96*



a. The typical "pile of coin" effect makes this easy to recognise ×270

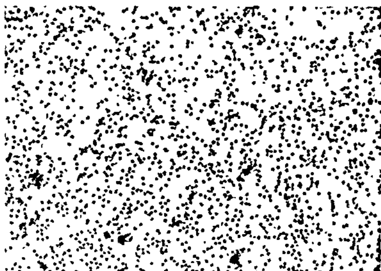


b. Here the "pile of coin" effect is less apparent than in *a* but can still be seen. ×270

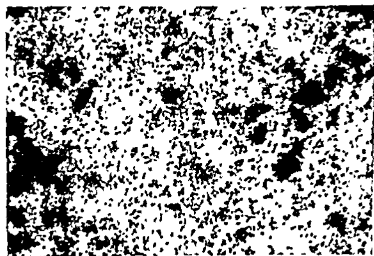


c. Rouleaux due to Dextran. ×270

PLATE X.



a. False agglutination due to fibrin particles in the serum to which the cells adhere See p 97 ×220



b False agglutination due to Wharton's jelly. See p. 98 ×220

up into smaller clumps before the typical appearance becomes visible. A further diagnostic point is that rouleaux formation is as strong at 37° C. as at 16° C.

In rouleaux formation due to Dextran, though the basic arrangement is still the same, a slightly different appearance is seen. The outline of the cells often becomes blurred so that discrete cells cannot be distinguished within the clumps. The appearance is quite typical and can be recognized with experience (Plate IXc).

On adding saline to the slide, the cells disperse readily. This is the most useful diagnostic point, but it should not be forgotten that strong rouleaux may only disperse partially with the amount of saline that it is feasible to place on a slide. Furthermore, at least one or two minutes is usually needed for the dispersal to take place. The process is accelerated by warming the slide, but this may lead to confusion with auto-agglutination.

Slight drying up of the cell suspension on the slide accentuates rouleaux. Lewis agglutination sometimes resembles rouleaux formation in appearance and the distinction between the two can occasionally be difficult.

The rouleaux forming factors in sera are not attached to the cells in any way and are readily removed from the cells by washing. There is thus no difficulty in preparing free cell suspensions and in testing them. For the same reason rouleaux do not give rise to any difficulties in the Coombs test. Indeed, when working with strongly rouleaux forming sera one has often to rely mainly on this test, which may be the only one whose interpretation is free from doubt.

5. Particles. Sera may contain particles of dust or other debris, or they may develop particles of fibrin or denatured protein on prolonged storage (Plate X*a*). Another change that sometimes occurs in sera on prolonged storage in the frozen state is that fats come out of solution or change to soaps, and globules form in the serum. The presence of particles in the sera is liable to lead to confusion because erythrocytes tend to stick to such particles and this can give an appearance resembling agglutination. Such sera should be freed from these particles by centrifugation.

6. Separation of Proteins due to Freezing. When serum freezes, the water freezes first and rises to the top, leaving a more concentrated protein solution at the bottom which freezes later. If the serum is subsequently thawed without mixing, two or more distinct layers may be seen in the liquid. Repeated freezing and thawing accentuates the phenomenon. This can cause either a false positive or a false negative result. If the pipette is placed in the top layer of the serum a watery solution containing very little antibody will be drawn up and a

false negative result will ensue. If the pipette is placed in the bottom layer, a viscous, sometimes even treacly, fluid will be withdrawn which can give rise to marked rouleaux formation. Such difficulties are easily avoided if one always remembers to mix tubes of serum before use, either by inverting the tube a few times or by drawing the contents up and down in the pipette. Shaking should be avoided since frothing causes denaturation of proteins.

7. WHARTON'S JELLY (Plate X*b*). Wharton's Jelly is the material lying between the vessels of the umbilical cord. It is sometimes an accidental contaminant in samples of cord blood. This arises from the incorrect practice of collecting the sample by "milking" the cord. The presence of Wharton's Jelly in cell suspensions causes an appearance which it is possible to mistake for agglutination, and we have known this to cause an error in determining the ABO group. Washing the cells is of no advantage, since the jelly is spun down with the cells each time they are washed. Wharton's Jelly can thus cause a false positive result in the direct Coombs test. Once the sample is contaminated with the jelly it is not very satisfactory to attempt to get rid of it, although this can sometimes be achieved by a process of differential centrifugation. The cells are suspended in a large volume of saline and centrifuged for a short time at a low speed. If this is done correctly it is possible to get the lumps of jelly spun down, leaving a jelly-free cell suspension at the top of the tube which can then be removed and used for testing. In some cases the jelly is not present as discrete lumps but the whole specimen is viscous and sticky, and we have not succeeded in preparing a satisfactory cell suspension from such a specimen. Contamination with Wharton's Jelly is a case where prevention is better than cure, and care should accordingly be taken in the collection of samples of cord blood.

8. UNSUSPECTED ANTIBODIES. The presence in sera of antibodies other than those which are thought to be present may give rise to false positive results. Standard antisera should always be thoroughly tested with a large panel of standard cells before they are put into use, but it is, nevertheless, always possible for an antibody to be overlooked or for some rare or unknown antibody to be present, and it should also be remembered that sera are usually tested with regard to their suitability for use by one particular technique and this may not necessarily mean that they would give specific results if used by some other technique. On one occasion, for example, we encountered an anti-A typing serum which contained a saline agglutinating anti-D, and when working with the rarer sera it is often a matter of considerable difficulty to make quite sure what antibodies sera do contain. A phenomenon associated with immune sera is the return of absorbed

antibodies to the serum. In Chapter II a technique of absorption has been described to minimize this. The commonest instance of this strange phenomenon is the reappearance of anti-A and anti-B in absorbed sera. We have seen this many times and sometimes the anti-A and anti-B are even strong enough to give a visual positive result. For this reason it is desirable to control the absorption of sera by testing with group A and group B cells of suitable other groups at frequent intervals.

The phenomenon is not confined to anti-A and anti-B and in the following case anti-D returned to the serum:

CASE 21

A serum contained the antibodies anti-C, anti-D and anti-Kell. The first two of these were removed from the serum by absorption in order to give a specific anti-Kell typing serum. This serum gave specific results for a time, but anti-D subsequently reappeared in it. In another case anti-D was absorbed from an anti-C+D serum, but subsequently reappeared.

The phenomenon can also occur with species specific antibodies present in immune animal sera, e.g. antiglobulin sera, anti-M, anti-N.

9. ACCIDENTAL MIXING OF SERA. This is a mistake commonly made by inexperienced technicians through not taking sufficient care to wash their pipettes out. On one occasion where difficulty was encountered in determining an ABO group, we found that the anti-A serum would agglutinate B cells and vice versa. It is desirable to have separate pipettes for anti-A and anti-B sera in order to avoid this error. The practice of issuing sera in bottles with indwelling pipettes has much to commend it.

FALSE POSITIVE RESULTS DUE TO ERRORS OF TECHNIQUE

1. CLOTS. Difficulties due to clots arise in two ways. Firstly, if cell suspensions are prepared from samples of clotted blood they will often contain small fragments of clot broken off when the suspensions were prepared, and these may be mistaken for agglutinates. After the cell suspension has been made from the clot it should be left standing on the bench for a few minutes in order that any fragments of clot present may settle to the bottom. When the suspension is used the pipette should not be placed in the bottom of the tube, but the suspension from the top of the tube should be used. These precautions will usually suffice to avoid this difficulty.

The second way in which clots can give rise to trouble is where clotting takes place during the actual performance of the test. If unwashed or inadequately washed cells from a citrated sample of blood are mixed with serum, the citrated plasma present may form a clot

whilst the tube is being incubated. When the contents of the tube are pipetted out in order to read the result of the test, all that is seen in such cases is a small clot in which all the cells are enmeshed. This can be mistaken for a massive agglutinate. Even if the clot is recognized for what it is, however, it is not safe merely to examine any cells which may remain free from the clot, since the formation of fibrin during the test has interfered with the sedimentation of the cells, and the test must be repeated using a more thoroughly washed cell suspension. In cases where the clot forms more slowly, the cells may have settled to the bottom of the tube before the clot has formed, and here a pale plasma clot will be seen, but this also is unsatisfactory. This phenomenon of clotting during the performance of the test can be most perplexing to those who do not know what they are dealing with. Occasionally when a transfusion is required urgently a sample of the patient's blood is taken and the serum separated so rapidly that the clotting process is not complete, and the patient's serum is still forming clot during the performance of the cross matching test.

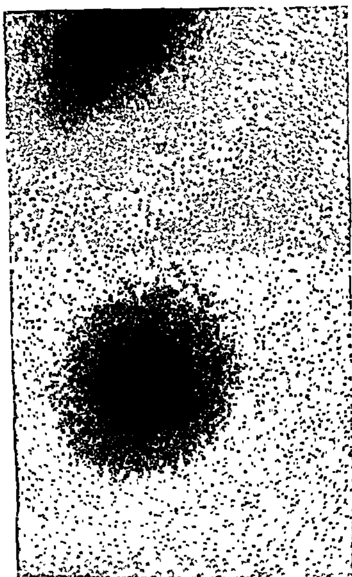
CASE 22

Blood was required for a patient who bled unexpectedly during the performance of a prostatectomy. The patient was group O, but owing to confusion with another patient of similar name was thought to be group A. A sample of blood was obtained whilst the patient was in the theatre, the serum separated immediately and cross matched against group A blood on a tile. Clotting occurred on the tile and was recognized as such, but the fact that agglutination was also occurring was overlooked, and an incompatible transfusion resulted.

2. COMETS (Plate XI). In agglutination tests which have been incubated for too long or which have been centrifuged excessively, an appearance superficially resembling agglutination may sometimes be seen. On pipetting the contents of the tube out on to a slide a number of aggregates are visible to the naked eye. Microscopical examination shows that these are not agglutinates but merely masses of cells in close juxtaposition. If the slide is tilted and a moving field examined it can be seen that cells are dislodged from the edge of the mass as the surrounding fluid flows past it. These dislodged cells trail away behind the mass, forming a tail. The whole appearance is somewhat reminiscent of its celestial namesake. A small amount of comet formation does not give rise to much difficulty in reading the test, but if centrifugation has been excessive and comets are numerous the picture becomes confusing.

3. INFECTED ALBUMIN. Albumin and serum albumin may become bacterially contaminated and behave in a similar manner to the infected serum considered above.

PLATE XI.



A "Comet." See p 100 ×220

4. **Dirty Glassware.** This has been discussed above.

5. **Poor Quality Saline.** The use of unsterile saline or saline containing particles of dust, fluff, etc., is a frequent cause of difficulty.

6. **Naked-eye Reading of Results.** On pipetting the contents of a tube on to a microscope slide agglutination is sometimes obvious to the naked eye, and it is tempting to record the result as positive without taking the trouble to look at it down a microscope. This is a dangerous practice. Most of the causes of false positive results considered above give an appearance which can only be differentiated from a true antibody agglutination by careful microscopical examination. One should always be suspicious of any agglutination showing an atypical appearance.

7. **Drying up.** When carrying out agglutination tests on microscope slides, these should not be left too long or drying up will interfere with the reading and may increase rouleaux formation. We consider that six minutes is the optimal time.

False Negative Results due to Factors affecting the Erythrocytes

1. **Deterioration of Antigen.** Quite apart from any question of bacterial contamination, the activity of the blood group antigens on the red cell surface gradually deteriorates when the cells are stored. It has been our experience that clotted samples received through the post normally give satisfactory results in ABO and Rh grouping tests. For special purposes, however, such as the investigation of weak forms of the antigens, the cells should be as fresh as possible. We have repeatedly observed that the reactions of D^u blood change slightly after only a day or two at 4° C. In identifying an unknown antibody it is most important to test it with a panel of cells which have all been collected at the same time and into the same anticoagulant, otherwise a confusing picture is likely to be seen when the results are read. If some of the cells of the panel are freshly collected and others have been preserved in a frozen state, discrepancies are very likely to occur. The autoagglutinin receptor on the red cell surface, in particular, does not seem to be well preserved in a frozen state, and we have several times

2. **Chemical Contamination.**

3. **Cell Mixtures.** These have been discussed already. They may cause either f

4. WEAK FORMS OF ANTIGEN. In certain cases the antigen exists in a weak form which may escape detection.

The A antigen of A_2B is the best known of these. A_3 and A_4 are even weaker forms of the antigen, and D^u is a commonly occurring weak form of the D antigen. The detection of these antigens is considered elsewhere.

5. BLOCKING OF ANTIGEN. In D-typing infants suffering from haemolytic disease, one sometimes gets a negative result with the agglutinating anti-D serum because the antigen is blocked by incomplete anti-D (see p. 196). In acquired haemolytic anaemia also, antibodies are sometimes present which are specific for antigens on the patient's own red cells, and these occasionally block the antigens against the corresponding antisera (p. 244).

FALSE NEGATIVE RESULTS DUE TO FACTORS AFFECTING THE SERUM

1. OMISSION OF SERUM FROM THE TUBE. In putting up a large number of tests at the same time, it is very easy for the serum to be accidentally missed from one of the tubes. If the cell suspensions have been placed in the tubes first, the fact that no serum has been added may be overlooked. It is better to put the serum in the tubes first and then inspect each tube to see that it contains serum before the cell suspensions are added.

2. CHEMICAL CONTAMINATION. This has been discussed already. An instance of it was the serum of a group O patient in which neither anti-A nor anti-B could be detected. This proved to be due to accidental contamination of the sample by phenol. Anti-A and anti-B were present in a second sample of this patient's blood.

3. DETERIORATION OF ANTIBODY. Most of the blood group antibodies are relatively unstable substances, some of them extremely so, and sera ought to be handled with a great deal more care than they generally receive. Sera which depend on complement for their action, notably those containing anti-Lewis and anti-Jk^a, are the most unstable of all, but even a saline agglutinating anti-Rh serum can become noticeably weaker after only a few hours at room temperature. All sera ought to be kept frozen when not in use. Furthermore, sera ought to be thawed out in the 37° C. and not in the 56° C. water bath. Two examples of the instability of anti-Le^a may be given:

CASE 23

P.S. required transfusion after a partial gastrectomy, but the hospital found an incompatibility in the Coombs test on cross matching. We were unable to detect any antibody in the sample of blood which we received. The sample had been unrefrigerated

for two days whilst in transit. Our report of "No antibody present" was received by the hospital with some scepticism and a second sample was sent and tested whilst in a fresh state. Anti-Le^a was then detected.

CASE 24

Anti-Le^a was detected in a fresh sample of serum but vanished when the serum was stored frozen over the week-end.

Very dilute sera are prone to lose their activity through surface effects. Protein molecules readily form mono-molecular layers on glass surfaces and at liquid-air interfaces, and this causes a loss of protein from the serum. This is why frothing of serum should be avoided. Shaking can also cause protein denaturation.

On one occasion we were investigating a rabbit anti-human-red-cell serum active at a dilution of 1 in 30,000. The action of this appeared to be potentiated by
but the results were e
(at a dilution of 1 in 30,000 in saline) were gently rolled round the surface of a clean glass tube and then transferred to a second and third tube, and the process repeated in each case, all activity was lost. The addition of human serum, even at a dilution of 1 in 1,000, prevented this effect. We attribute this phenomenon to loss of antibody from the serum by adsorption on to the glass surface of the tubes, and we suppose that when human serum is added the protein molecules in this compete with the rabbit antibody for the glass surface so that there is less loss of antibody.

If it is desired to use a serum in high dilution some inert protein should be added; normal serum or haemoglobin is suitable. We have encountered the same phenomenon with very dilute solutions of human γ-globulin whose activity was measured by means of their power to inhibit Coombs reagent. Loss of activity in these solutions was satisfactorily prevented by the addition of human haemoglobin. The same thing is also likely to occur with antibody eluates which frequently contain only very small amounts of protein. For this reason we consider some haemolysis as an advantage when eluates are made by heating, since the haemoglobin liberated will stabilize the eluate.

Another instance of surface effects is sometimes seen in the Coombs test. Washed cells will adhere to a clean glass surface, but this is prevented if the surface is first exposed to serum at a dilution stronger than about 1 in 500. Thus it is difficult to centrifuge serum-free cell suspensions in clean tubes in an angle head centrifuge owing to the cells sticking to the glass. In washing cells for the Coombs test, however, the effect does not ordinarily occur, since the walls of the tube take up a layer of protein which they retain during the subsequent washing. In carrying out a Coombs test using an eluate instead of

serum, or using very small amounts or high dilutions of sera, there is apt to be a considerable loss of cells during washing through sticking to the sides of the tube.

4. SEPARATION OF PROTEINS DUE TO FREEZING. This has been discussed (p. 97).

5. HAEMOLYSINS. In fresh serum, particularly of group O, anti-A and anti-B are not infrequently active as specific haemolysins. It is sometimes stated that haemolysis can be taken as the equivalent of agglutination in the determination of the ABO group and recorded as a positive result. This is unwise, however, since one generally does not know whether the haemolysis was due to a specific haemolysin or to some accidental mistake in the technique. It is better to destroy complement by inactivating the serum at 56° C. for half an hour and then repeat the test, when the expected agglutination should be observed. Anti-A and anti-B haemolysins are occasionally strong enough to cause total lysis even when the test is carried out on a slide or tile (Plate XII). If this happens and the test has been performed on a white tile, it is possible to mistake the uniform pink colour of complete lysis for a negative result. This is one of the reasons why the test should not be carried out on a white tile. If it is carried out on a microscope slide, microscopical examination will avoid this difficulty. Rh antibodies never cause in vitro lysis.

6. ZONING. Prozones are very rare with anti-A and anti-B but occur more frequently with Rh antibodies. Anti-Rh agglutinins showing zoning are encountered from time to time, and this is also sometimes seen with albumin suspensions of red cells. Zoning is less marked with serum-albumin suspensions than with albumin suspensions.

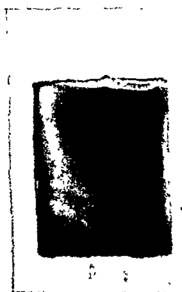
CASE 25

A patient narrowly escaped an incompatible transfusion from this cause; the cross matching test was declared incompatible on the grounds of a weakly positive albumin test which might easily have been missed. No Coombs test had been done. The weak results in the albumin test in this case certainly gave no indication of the very strong incomplete anti-D which was present. When the serum was diluted, a strong result in albumin was obtained.

CASE 26

Another instance of difficulty due to zoning with Rh antibodies occurred with an anti-C serum. This serum was a saline anti-C plus incomplete anti-D serum. It was found to give negative results with both R_2r and R_2R_2 cells, but was considered unfit for use since it agglutinated R_2r cells in saline suspension at a dilution of 1 in 8.

PLATE XII. Four stages in the action of an anti-A haemolysin. See p. 104. × 1½



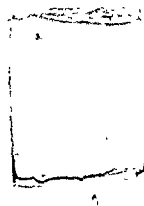
a. 0 minutes—no agglutination.



b. 2 minutes—agglutination.

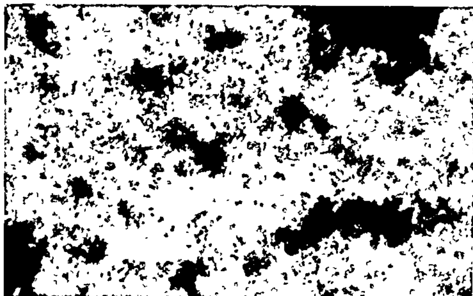


c. 5 minutes—agglutination dispersing.



d. 7 minutes—cells almost completely lysed.

PLATE XIII.



a Agglutination due to saline anti-D. ×220



b Weak agglutination due to excessively thick cell suspension (same serum as (*a*)) See p 105 ×220



c Weak agglutination due to clumsy technique in reading test (same serum and cells as in (*a*)) See p 105. ×220

We have never encountered an Rh antibody showing zoning in the Coombs test, but the antiglobulin reagent used in this test sometimes shows zoning itself, and this is a well-known cause of difficulty (p. 68).

7. ABSENCE OR WEAKNESS OF ANTI-A AND ANTI-B. In the sera of newborn infants anti-A and anti-B are always weak and frequently absent (p. 107); this can cause difficulties in ABO grouping. They are also sometimes weak in old age. Three very rare circumstances in which they may be absent are agammaglobulinaemia, the chimera (p. 3), and where weak forms of the A and B antigens are present (p. 111).

FALSE NEGATIVE RESULTS DUE TO FACTORS AFFECTING THE TECHNIQUE

1. THICKNESS OF CELL SUSPENSION. The use of too thick a cell suspension is an important cause of false negative results (p. 31). Plates XIII (*a*) and (*b*) show this in the case of an anti-Rh serum.

2. DIRTY GLASSWARE. This has been discussed (p. 89).

3. HAEMOLYSIS. Specific haemolysins have been considered above, but haemolysis in the test may also occur from a variety of accidental causes. Perhaps the tube was contaminated by soap or other chemical substance, or perhaps some water has been splashed into the tube during incubation, or a droplet of condensate has fallen into the tube from the lid of the water bath. The presence of haemolysis indicates either that something untoward has occurred during the performance of the test, and the test must be repeated, or that a specific haemolysin is present. Even though the haemolysis is only partial, it is unsafe to read the result by inspecting the cells which remain; we have encountered a false negative result from this cause on a number of occasions.

4. INCORRECT TEMPERATURE OR TIME OF INCUBATION. It is important to carry [illegible] ect time according t[illegible] is being allowed tc [illegible] temperature during the whole of the period of the test. Placing the tubes in a 37° C. water bath will ensure this, but if this is inconvenient the cold tubes containing the reagents should be placed in a warm rack in the incubator. Neglect of this precaution is a frequent cause of difficulty in Rh typing.

5. POOR TECHNIQUE IN READING RESULTS. Insufficient care in reading the results and lack of attention to the correct technique (p. 33) are important causes of false negative results with inexperienced workers (Plate XIII (*a*) and (*c*)).

Other Causes of False Positive and False Negative Results

We discuss elsewhere some further causes of false positive and false negative results which occur in the serum albumin test (p. 35), the papain cell test (p. 44), the antiglobulin test (p. 72), and in MN (p. 285) and Lewis grouping (p. 299).

References

Chown, B. (1954). Anaemia from bleeding of the foetus into the maternal circulation. *Lancet*, i, 1213.

Chown, B. (1955). The foetus can bleed. *Am. J. Obstet. and Gynaecol.*, **70**, 1298.

Davidsohn, I., and Toharsky, B. (1940). The production of bacteriogenic hemagglutination. *J. Infec. Dis.*, **67**, 25.

Dunsford, I. (1957). Proof of foetal antigens entering the maternal circulation. *Vox Sang.*, **2**, 125.

Moskowitz, M., and Carb, S. (1957). Surface alteration and the agglutinability of red cells. *Nature*, **180**, 1049.

Moskowitz, M., and Treffers, H. P. (1950). An agglutinin in normal sera for periodate-treated red cells. *Science*, **111**, 717.

Stratton, F. (1943). Some observations on autohaemagglutination. *Lancet*, i, 613.

Stratton, F. (1954). Polyagglutinability of red cells. *Vox Sang.*, **4**, 58.

Stratton, F., Renton, P. H., and Hancock, Jeanne A. (1958). Red cell agglutinability affected by disease. *Nature*, **181**, 62.

CHAPTER VI

THE ABO GROUPS

The Four Main Groups

THE ABO group of an individual is determined by the presence or absence on the surface of his erythrocytes of the antigens A and B. These antigens react with corresponding agglutinins in the serum of persons whose red cells do not bear the corresponding antigens. These reactions are shown in Table 31.

TABLE 31. THE ABO GROUPS

Group	*Antigens on cells*	*Antibodies in serum*
A	A	Anti-B
B	B	Anti-A
AB	A and B	—
O	—	*Anti-A and Anti-B*

The agglutinins, anti-A and anti-B, act best against saline suspensions of red cells, and they almost always act best at low temperatures. The reactions are often weak or even absent at 37° C. but are almost always satisfactory at 16° C. The titres of the anti-A and anti-B agglutinins vary in different individuals and at different periods of the individual's life, tending to be low in childhood and in old age. The anti-B titre is generally lower than the anti-A, and in old age anti-B is occasionally so weak as to be only demonstrable at refrigerator temperature or by means of enzyme treated cells. In newborn infants the agglutinins are weak and are sometimes absent, according to the rule that a newborn infant's serum will never agglutinate its mother's cells. For example, the serum of the group O infant of a group A mother may contain anti-B but not anti-A. The so-called "defective" groups, where the expected anti-A or anti-B is absent, are of doubtful existence. Defective groups were described by some of the earlier workers, but in our experience anti-A and anti-B are only absent where the corresponding antigen is present, except in newborn infants and cases of agammaglobulinaemia. All the defective groups examined recently have been shown on further investigation to be cases where either the agglutinins were present but weak, or cases where weak forms of the A and B antigens were present: A_3, A_4, etc. We have never seen a true defective group ourselves.

The Subgroups

The A antigen in all samples of group A (or AB) blood is not identical. The first distinction to be made was that between A_1 (and A_1B)

and A_2 (A_2B), and this distinction is made by means of the reagent anti-A_1. The subgroups A_1 and A_2 remain much the commonest and most important of the subgroups of A, though weaker and much rarer subgroups of A and some rare subgroups of B are also known. The main reactions of the subgroups of A are summarized in Table 32.

TABLE 32. REACTIONS OF SUBGROUPS OF A

Subgroup	*Reagent*			*Remarks*
	Anti-A_1	*Anti-A*	*Group O serum*	
A_1 ..	+	+	+	
A_2 ..	−	+	+	
A_3 ..	−	+	+	Reactions weaker than A_2. Agglutination sometimes has typical appearance.
A_4 ..	−	−	+	Certain Group O sera only.
A_m ..	−	−	−	A substance in secretions.
A_0, A_x, A_5, A_z, A_4				Similar to A_4.

A_1 AND A_2 (see Wiener, 1948)

About two in nine A bloods are A_2, and about one in four AB bloods are A_2B. The distinction between A_1 and A_2 is drawn by means of the antibody anti-A_1 which is of occasional natural occurrence in the serum of A_2 and A_2B persons, and can also be prepared artificially by absorbing group B serum with A_2 cells. The naturally occurring anti-A_1 is found more frequently in A_2B persons than in A_2 persons. It acts best at low temperatures and is inactive at 37° C. In our experience it is usually of low titre, and naturally occurring examples of anti-A_1 are scarcely ever of any value as typing sera.

Von Dungern and Hirszfeld (see Wiener, 1948, p. 203) showed that, when a group B serum is absorbed with progressively increasing amounts of A_2 cells, the reaction with A_2 cells is abolished first, leaving a reagent specific in its reactions with A_1 cells. Further absorption will, however, remove the reaction with A_1 cells also. If a group B serum is absorbed with A_1 cells the same sequence of events takes place, but much smaller amounts of A_1 than of A_2 cells are needed. This phenomenon shows that there is a cross reaction between the antigens and antibodies concerned.

The main practical importance of these subgroups lies in the fact that the weakly reacting A_2 antigen may not be detected in ABO grouping. There is not usually much danger of this occurring with A_2 cells, but it is a very real danger with A_2B cells. The reason for this is that the

reaction of A_2 with anti-A typing serum is not much weaker than with A_1, but the reaction of A_2B is often considerably weaker if the typing serum is from non-immunized individuals. This constitutes a well known and very important source of error in ABO grouping, and it is essential that anti-A typing serum should give good results with A_2B cells. The occasional presence of anti-A_1 in the serum of A_2B bloods is a further source of difficulty, and combined with the weakness of the A antigen makes them liable to be mis-diagnosed as B.

The A antigen is not fully developed at birth so that attempts to differentiate A_1 from A_2 are usually unsuccessful. Difficulty in determing the ABO group is also occasionally encountered for the same reason.

A_3 (Friedenreich, 1936; Gammelgaard, 1942)

A_3 and A_3B cells react with anti-A sera very much more weakly than do A_2 and A_2B cells, but if the test is carried out in tubes there is no doubt that they do give a clear-cut positive result with anti-A sera, though the agglutination is weaker and the titre of the serum less than with A_1. Some A[illegible] bloods present a [illegible] description may appear somewhat vague there is, nevertheless, in practice no difficulty in making the distinction between A_2 and A_3. The results obtained on titrating an anti-A serum with A_3B cells are shown in Table 33. The frequency of A_3 is about one per thousand A bloods. No special reagents are required for the demonstration of A_3.

TABLE 33. TITRATION OF ANTI-A WITH A_2B AND A_3B CELLS

Cells	Titre of anti-A serum								
	1	2	4	8	16	32	64	128	256
A_3B	+++	++	+	w	—	—	—	—	—
A_2B	v	v	+++	+++	++	++	w	—	—

A_4 AND OTHER WEAK A ANTIGENS (see Race, 1957)

A_4 is a still weaker form of the A antigen (Fischer and Hahn, 1935; Gammelgaard and Marcussen, 1940; Gammelgaard, 1942; Dunsford, 1952; Dunsford and Aspinall, 1952; Estola and Elo, 1952; Bell, 1953). Its frequency is thought to be between 1 in 8,000 and 1 in 40,000 white bloods (Dunsford, 1955), although it is said to be more common in Negroes. A_4 is recognized by the fact that it is agglutinated by group O but not by group B sera, although a weak reaction with group B sera can be demonstrated by elution. Furthermore, using fresh samples and

testing carefully at a low temperature, it is often possible to demonstrate a weak agglutination with certain group B sera. For all practical purposes, however, blood which gives a clear-cut agglutination with a proportion of group O sera and little or no agglutination with group B sera is considered to be A_4.

If A_4 cells are treated with group O serum and the antibody which becomes absorbed on to them is recovered by elution, it will be found to react not only with A cells but with B cells also (Table 34), and it has been suggested (Wiener, 1953) that the antigen on A_4 cells represents a common component between A and B cells (sometimes called C).

TABLE 34 REACTIONS OF ELUATE OF A_4 CELLS AGGLUTINATED BY GROUP O SERUM

	Saline agglutinations at 16° *C.*				
	A_1	A_2	A_4	O	B
Eluate . . .	v	+++	++	—	v
Serum	v	v	+++	—	v

According to this theory, A is really AC, B is really BC, A_4 is C alone, *and O has none of the three antigens. Anti-A and anti-B from group* B and group A persons respectively supposedly do not contain anti-C, but group O serum is supposed to contain anti-A, anti-B and anti-C. The so-called anti-C appears to be a cross-reacting component within the group O serum, and it can be shown that absorption of group O serum with group A cells will often lower its titre with group B cells and vice versa. Furthermore, an eluate of group A cells agglutinated by group O serum will often agglutinate group B cells and vice versa. Whilst there is probably an element of truth in this theory it cannot be the complete explanation, since there is no doubt that A_4 cells do react to some extent with group B sera. Only certain group O sera give good results with A_4 cells; these are often those which show immune characteristics.

The practical importance of A_4 has, in our opinion, been greatly over-emphasized. In the first place, it is of very infrequent occurrence in the white population, and in the second place, it is very doubtful whether any A_4 blood samples remaining undetected would pass the cross-matching test. The use of group O serum in ABO grouping for the purpose of detecting A_4 blood, therefore, hardly seems worth while. On the other hand, the use of group O serum does provide a valuable check on the accuracy of the test with anti-A and anti-B sera, and is probably worth using for this purpose. However, perhaps the argument is rather academic. The detection of A_4B appears to be very difficult,

except by family studies, since group O serum cannot be used for obvious reasons, and group O serum absorbed with B cells may fail to react with A_4 cells (Table 35). Eluates of A_4 cells agglutinated by group O serum react with B cells (Table 34), but it might be possible to demonstrate the A_4 antigen in A_4B cells by preparing an eluate using group B serum with the A_4B cells, and showing that the eluate would agglutinate A_1 cells. Elution is a sensitive means of demonstrating the reactions of these weak A antigens.

TABLE 35. REACTIONS OF GROUP O SERA ABSORBED WITH GROUP B CELLS

Group O serum	*Absorption*	*Saline agglutinations at 16° C.*				
		A_1	A_2	A_4	O	B
No. 56	×1 with B cells	v	v	+++	–	+
	×2 with B cells	v	v	–	–	–
	×1 with O cells (control) ..	v	v	v	–	v
	×2 with O cells (control) ..	v	v	v	–	v
No. 67	×1 with B cells	v	v	++	–	–
	×2 with B cells	v	v	–	–	–
	×1 with O cells (control) ..	v	v	v	–	v
	×2 with O cells (control) ..	v	v	+++	–	v

Bloods described as A_0, A_x, A_5, A_z and A_g appear to differ very little, if at all, in their reactions from A_4 (Gammelgaard, 1942; Grove-Rasmussen *et al.*, 1952; Engelfried, 1955; Ellis and Cawley, 1957; van Loghem *et al.*, 1957).

The type of blood called A_m (Wiener and Gordon, 1956) has no detectable A antigen on the red cell. It is demonstrated by the absence of anti-A in the serum and by the presence of A substance in the secretions.

There is evidence that some of these weaker reacting A bloods are inherited by means of an allele at the ABO locus, but in other cases the inheritance appears to depend on the effect of modifying genes (van Loghem *et al.*, 1954; Beckers *et al.*, 1955; Cahan *et al.*, 1957; Weiner *et al.*, 1957). There is even some evidence that the reactions of the A antigen can very rarely become modified during a patient's lifetime, as shown by van Loghem *et al.* (1957) and the following case (Stratton *et al.*, 1958).

CASE 27

The cells of S.M., a case of hypoplastic anaemia, gave the following reactions in the ABO grouping test:

Anti-A	*Anti-B*	*A cells*	*B cells*
–	–	–	v

Further tests failed to show the presence of any anti-A in his serum, but his cells were agglutinated by 28 out of 70 group O sera at 16° C., and anti-A could be eluted from his cells when they were treated with either group B or group O serum. The reactions were weaker than those of A_4 cells. It was subsequently found that his wife was group O and his daughter a normal A_1, and that he had been found a normal group A when grouped in the army some years previously. His A antigen thus appears to have changed during his lifetime.

Weak B Antigens

These seem to be very rare. The first two described were named B_3 (Moullec *et al.*, 1955) and B_w (Levine *et al.*, 1956), and reacted in a manner somewhat analogous to A_3. The third, B_x, was a still weaker variant, probably analogous to A_m (Dunsford *et al.*, 1956). No variant of B reacting like A_2 has been described. We have seen two examples of weak B antigens ourselves:

Case 28

Mrs. B. L. appeared to be $A_1B_?$. Her A antigen was normal and her serum contained an apparently normal anti-B but no anti-A. Her cells were agglutinated at 16° C. by 13 out of 52 group A sera, and this agglutination was inhibited by the saliva of group B individuals only. Her cells were not agglutinated by 36 group AB sera.

Case 29

Mr. H. R. also appeared to be $A_1B_?$ with a normal A antigen and apparently normal anti-B in the serum. His cells were agglutinated by 34 out of 36 group A sera at 16° C. The reactions varied from + to +++ in strength and were inhibited by group B saliva only. His cells were not agglutinated by 25 group AB sera. The reactions in this case were stronger than in the preceding one.

The Preparation of ABO Typing Serum

Anti-A, Anti-B and Group O (Anti-A+B) Serum

Sera containing anti-A and anti-B [illegible] may [illegible] tain [illegible] of volunteers with A or B [illegible] latter procedure probably produces the better reagent, and in many cases the sera of immunized volunteers have much better titres and avidities than those of non-immunized persons.

The suitability of a serum for use as a grouping serum depends on a number of characteristics, all of which must be satisfactory. These comprise:

1. Titre.
2. Avidity (measured by speed of reaction).
3. Firmness of agglutinates formed.

4. Reaction with A_2B cells.
5. Sterility.
6. Freedom from rouleaux formation.
7. Specificity.
8. Freedom from fats and particles.
9. The serum must not contain an active haemolysin.

Suitable requirements for the first four of these characteristics are shown in Table 36 and in this table the titres are determined against 3 per cent saline suspensions of washed cells by the tube technique, reading microscopically after two hours' incubation at room temperature. The avidity is determined by mixing one drop of a 10 per cent saline suspension of washed cells with one drop of serum on a microscope slide, and rocking gently. The time from mixing to the first naked-eye appearance of agglutination is noted.

The reaction with A_2B cells is particularly important, since the A antigen of A_2B cells is a good deal weaker even than that of A_2 cells, and the serum can give satisfactory results with A_1 and A_2 and have a good titre without it necessarily meaning that the reaction with A_2B cells will be satisfactory.

TABLE 36. MINIMAL REQUIREMENTS FOR GROUPING SERA

Serum	*Test cells*	*Titre*	*Avidity*	*Firmness of agglutination*
Anti-A ..	A_1	64	10 secs.	+++++ at 1 in 4
	A_2	32	20 secs.	
	A_2B	8	30 secs.	
Anti-B ..	B	64	10 secs.	+++++ at 1 in 4

Group O sera (anti-A+B) should combine the characteristics required for anti-A and anti-B and should also give a ++++ agglutination with A_4 cells when tested by the tube technique reading microscopically.

The sterility of the reagent is important, since infected reagents can give rise to very deceptive false positive results. We have seen an infected anti-A serum which agglutinated group O cells on a slide at room temperature just as rapidly and just as intensely as group A cells; indeed the two reactions were indistinguishable. The best means of ensuring the sterility of the serum is to handle it aseptically during processing. Seitz filtration is undesirable, since it reduces the titre and avidity. Some people consider it desirable to sterilize the serum by a process of Tyndallization in which the serum is heated to 56° C. for thirty minutes on two successive days, and this has the additional advantage of destroying complement and thus preventing any possibility of the anti-A or anti-B acting as a haemolysin when the serum is used.

The specificity of the serum is assessed by tests in tubes at 16° C. and 37° C. against saline suspensions of cells covering as many of the known blood group antigens as possible. Autoagglutinins, anti-P, anti-H and anti-O are the contaminants most frequently encountered, but anti-Rh, anti-Le^a, anti-Le^b and other antibodies are occasionally seen. Anti-B sera should be tested for anti-A_1, and ideally it would be desirable, though perhaps it is scarcely practicable, to test for anti-Wr^a, anti-Mi^a and anti-C^x, all of which have been found as naturally occurring antibodies. Anti-Wr^a is present in 1 per cent or more of normal sera but is not a frequent cause of false positive results with grouping sera owing to the low frequency (0·3 per cent) of the Wr^a antigen. A false positive result from this cause is to be anticipated about once in thirty thousand tests (1%×0·3%).

PREPARATION OF ANTI-A_1

Naturally occurring anti-A_1 sera, described above, are almost always unsuitable for use as anti-A_1 typing sera owing to their weakness, and it is preferable to prepare this reagent by the absorption of group B serum with A_2 cells. This is a process which requires a little care if a good result is to be obtained. The first step is to select a suitable group B serum for absorption; a good reagent cannot be produced from an unsuitable serum. Group B sera behave as though they contain two antibodies, anti-A which reacts almost as strongly with A_2 as with A_1, and anti-A_1 which reacts strongly with A_1 and only weakly with A_2 cells. The proportions of these two antibodies vary in the serum of different individuals, and it is essential to obtain a serum containing a high proportion of anti-A_1. The first step, therefore, is to titrate a number of group B sera against A_1 and A_2 cells, when it will be found that many give the same titre with both types of cells. Others show only a slight difference, and a few give a higher titre with A_1 than with A_2 by two or more tubes (doubling dilutions). Sera of the last type are suitable for the preparation of anti-A_1.

An alternative method of selecting suitable sera is by partial neutralization with A substance. Dilutions of an A secretor saliva are cautiously added to the sera and those sera are selected which give the best reactions with A_1 cells when the reaction with A_2 cells has just been abolished.

Preparation of the reagent is carried out by removing the anti-A from the serum by absorption with A_2 cells, and the difficulty here is to determine the appropriate proportion of cells to serum to use in the absorption. If too small a volume of cells is used the absorbed serum will still react weakly with A_2 cells, whilst over-absorption will weaken or even completely abolish the reactions with A_1 cells. The absorption may be conveniently carried out using washed cells and allowing cells

and serum to react for half an hour at room temperature. In order to determine the correct amount of cells to use, trial absorptions must first be carried out, and small portions of the serum should be absorbed with, for example, three-quarters, a half, and a quarter volume of packed cells. The absorbed sera are then tested with A_1, A_2, A_1B and A_2B cells on slides at room temperature, and a volume of cells selected which appears to give the best results. The bulk of the serum is then absorbed with the appropriate volume of cells for the same time at the same temperature. It should be remembered that, owing to the fact that antigen and antibody can combine in variable proportions, two successive absorptions with a quarter volume of cells would not produce the same result as one absorption with half a volume of cells.

Methods of ABO Typing

Whatever method is used, the two most important points are:

1. Test the patient's serum against A and B cells, as well as testing the patient's cells with anti-A and anti-B.
2. Carry out the test at room temperature or better at 16° C.

1. TUBE METHOD, READING MICROSCOPICALLY. Place one volume (0·04 ml.) of serum in a precipitin ($2\times\frac{1}{4}$ inch) tube and add one volume of a 3 per cent suspension of red cells, tap the tube to mix the contents, incubate for one and a half hours at room temperature (or better still, in a 16° C. incubator) and read the results by gently removing the contents of the tube with a Pasteur pipette on to a microscope slide and examining microscopically for the presence of agglutination.

We regard this as the most reliable method of all, but the long period of incubation makes the test rather slow without conferring a great increase of accuracy over method 3. This is a disadvantage in grouping prior to cross matching. If large numbers of groups have to be determined at the same time the process of reading the results microscopically is rather slow and tedious and method 2 may be preferred.

2. TUBE METHOD, READING BY TAPPING. Place 1 volume (0·08 ml.) of serum in a $2\times\frac{3}{8}$ inch tube and add 1 volume of a 3 per cent suspension of red cells. Tap the tube to mix the contents; incubate for one and a half hours at room temperature (or better still in a 16° C. incubator) and read the results by gently tapping the tube to dislodge the cells from the bottom, and inspecting naked eye.

In this method an increase of accuracy has been obtained by increasing the diameter of the tube (a $2\times\frac{3}{8}$ inch tube rather than a $2\times\frac{1}{4}$ inch tube as used in other agglutination tests), and by increasing the volumes of the serum and cell suspension (0·08 ml. of each rather than 0·04 ml. as in other agglutination tests). These measures make the test much

easier to read with the naked eye than is the case when smaller volumes and narrower tubes are used, and we consider them an essential part of the method.

This is probably the best method for large-scale grouping, combining a good standard of accuracy with ease of carrying out the procedures. There is, however, a slight loss of accuracy through not reading the results microscopically.

The use of a hand lens is, surprising as it may seem, a disadvantage. The reason for this is that the diameter of a red cell (7μ) is not a great deal less than the limit of visual acuity (about 30μ under the best conditions). Under certain oblique lighting conditions producing an element of dark-ground illumination a fine granularity due to the cells themselves can just be perceived in a red cell suspension and this is sometimes confused with a weak agglutination. Magnification increases the granularity and makes the distinction more difficult.

3. SLIDE METHOD. One volume (0·03 ml.) of serum is placed on a microscope slide with one volume of a 5 per cent suspension of cells, the drops are mixed, and the slide held in the hand with a gentle rocking motion for six minutes, after which the results are read by inspecting the slide, with the naked eye and microscopically.

It should be particularly noticed that a distinction is drawn between slide grouping and tile grouping. If the test is carried out on an opalescent white tile, no microscopic examination is possible. This method is, therefore to be condemned.

If done with care and read after a correct time interval it is doubtful whether this method is any less accurate than the preceding ones, and it is probably the best method for experienced workers to determine the ABO groups of patients where time is important. In inexperienced hands, the technique tends to degenerate, and we have seen, for example, people reading the test after as short a time as twenty seconds and only reading it with the naked eye. The method must be strictly followed if success is to be achieved. This is not a good method for large-scale grouping, since it is very difficult to keep a large number of microscope slides in the correct order and to read the test after the correct time interval.

USE OF GROUP O SERUM. Group O serum (anti-A+B) may be used in addition to anti-A and anti-B in testing the cells. This serves to detect A_4 though, in view of the very low frequency of this group, this is less important than its other function which is to serve as a check on the accuracy of the results with anti-A and anti-B.

The value of group O serum is greatest in large-scale ABO grouping using the tube method and reading by tapping. It serves here to compensate to some extent for the slight loss of accuracy entailed in not

reading the results microscopically and it also protects against some of the other errors which may occur.

In small-scale grouping, where the results are read microscopically and where greater care can be given to each individual test, it is less valuable. Errors of procedure are less likely here and the use of group O serum tends to some extent to divert attention from the really vital part of the test, which is to test the patient's serum as well as his cells. As shown below, these two tests check one another in such a peculiarly efficient manner that further tests are unnecessary if errors of procedure can be ruled out.

CONTROLS

Provided anti-A and anti-B grouping sera of known potency and specificity (p. 112) are used, the only control necessary in ABO grouping is to test the patient's serum against known A and B cells as well as testing his cells against anti-A and anti-B typing sera. The reciprocal relationship which exists between the iso-agglutinins and the antigens of the ABO system is such that this constitutes the most perfect control possible. Consideration of the possible causes of false positive and false negative results (enumerated in Chapter V) shows that none of them will fail to be detected by this control. It cannot be emphasized too strongly that it is absolutely essential to test the serum as well as the cells; indeed, we would go so far as to say that to do so is the most important precaution in the whole field of blood grouping work. The reason why this particular control is so very important and valuable is that by testing the serum as well as the cells, the presence or absence of the A or B antigen is always diagnosed by both a positive and a negative result, so that one has to have a false positive result in one half of the test combined with a false negative in the other half of the test before a mistake can be made. Many causes of false positive and false negative results are considered in this book, and doubtless others exist which are unknown to us, but it is hard to conceive of any single one capable of causing a false positive result in one half of a test combined with a false negative result in the other half of the test. This is why this particular control is so efficacious. The only way in which a mistake from serological causes (as opposed to failure of the human element) can occur, using this control, is where more than one of the possible causes of false positive and false negative results exist on the same occasion. Apart from the well-known fallacy caused by A_2B with anti-A_1 in the serum, this must be excessively rare and we have only known it once. On this occasion a group O blood was misdiagnosed as A. The false agglutination of the cells by anti-A serum was due to polyagglutinability and the false negative result when the patient's serum was tested with group A cells was due to haemolysis from a specific anti-A haemolysin being misread as a negative result.

PROCEDURE WHEN DIFFICULTIES OCCUR

Whilst testing the serum as well as the cells should suffice to prevent mistakes, more extensive tests will be needed to reveal the true ABO group once difficulty has been encountered, and in such cases it is best in the first place to repeat the test, testing the cells with anti-A, anti-B and AB serum, and the serum with A, B, O and the patient's own cells. All the results should be read microscopically after one and a half hours' incubation in tubes at 16° C. Alternatively, if one is in a hurry, the repeat test can be done by the slide technique, although a final test in tubes should always be carried out when the nature of the difficulty has been elucidated. If the difficulty was due to a mistake, a dirty tube, or inaccurate reading of the results in the first place, it often happens that no difficulty is encountered when the test is repeated in this manner. In other cases the results obtained by this test will give a useful indication of the probable nature of the difficulty.

The commonest causes of difficulty in ABO groupings are:

Errors of procedure.
Autoagglutinins.
Rouleaux.
Haemolysins.
Infected samples or infected reagents.
Anti-P, anti-A_1 or other antibodies in the patient's serum.

The best method of resolving these difficulties depends on the circumstances of the individual case, and this may best be illustrated by means of the following examples:

Example 1

First test.

Patient's cells		*Patient's serum*	
Anti-A	*Anti-B*	*A cells*	*B cells*
v	v	v	v

Repeat test.

Patient's cells			*Patient's serum*			*Patient's cells with patient's serum*
Anti-A	*Anti-B*	*AB serum*	*A cells*	*B cells*	*O cells*	
v	v	v	v	v	v	v

Remarks: Own cells agglutinated by own serum.
This dispersed on warming the slide, suggesting an autoagglutinin.

Cells washed at 37° C. yielding a free cell suspension which was tested at two temperatures as follows:

Final test.

	Patient's cells			*Patient's serum*			*Patient's cells with patient's serum*
	Anti-A	*Anti-B*	*AB serum*	*A cells*	*B cells*	*O cells*	
Test at 16° C.	–	v	–	v	v	v	v
Test at 37° C.	–	v	–	v	+	+	w

Conclusion Group B: Difficulty due to autoagglutinin: the serum could only be tested satisfactorily at 37° C.

Example 2

First test.

Patient's cells		*Patient's serum*	
Anti-A	*Anti-B*	*A cells*	*B cells*
v	–	++	+++

Repeat test.

Patient's cells			*Patient's serum*			*Patient's cells with patient's serum*
Anti-A	*Anti-B*	*AB serum*	*A cells*	*B cells*	*O cells*	
v	–	–	++	+++	+++	–

Remarks: Serum agglutinates O cells but not own cells, probably due to a specific antibody.
Further tests as outlined in Chapter XII showed anti-P in the patient's serum.

Final test.

Patient's cells			*Patient's serum*			*Patient's cells with patient's serum*
Anti-A	*Anti-B*	*AB serum*	*A P neg. cells*	*B P neg. cells*	*O P neg. cells*	
v	–	–	–	+++	–	–

Conclusion Group A: Difficulties due to anti-P in the serum.

Example 3

First test.

Patient's cells		*Patient's serum*	
Anti-A	*Anti-B*	*A cells*	*B cells*
++	–	+++	++

Repeat test.

Patient's cells			*Patient's serum*			*Patient's cells with patient's serum*
Anti-A	*Anti-B*	*AB serum*	*A cells*	*B cells*	*O cells*	
++	–	–	+++	++	–	–

Remarks: There is a discrepancy between the reaction of the cells with anti-A and of the serum with A cells.
Test more extensively using several different anti-A sera and several different A cell suspensions.

Further test.

Patient's cells with different anti-A sera					*Patient's serum with different A cells*				
1	2	3	4	*Anti-*A_1	A_1	A_1	A_1	A_2	A_1
++	–	–	–	–	+++	+++	+++	++	+++

Remarks: Anti-A No. 1 appears to be at fault.
Further tests showed that it agglutinated Group O cells, and it was found to be infected.

Conclusion Group O: Infected typing serum.

Example 4

First test.

Patient's cells		*Patient's serum*	
Anti-A	*Anti-B*	*A cells*	*B cells*
+++	–	++	+++

Repeat test.

Patient's cells			*Patient's serum*			*Patient's cells with patient's serum*
Anti-A	*Anti-B*	*AB serum*	*A cells*	*B cells*	*O cells*	
+++	–	–	++	+++	–	–

Remarks: Discrepancy between results with anti-A and with A cells. Test more extensively.

Further test.

Patient's cells with different anti-A sera					Patient's serum with different A cells				
1	2	3	4	Anti-A_1	A_1	A_1	A_1	A_2	A_2
+++	++	+++	++++	−	++	+++	++	−	−

Conclusion Group A_2: Anti-A_1 present.

TYPING WITH ANTI-A_1

Anti-A_1 typing serum is best used on slides, reading the results with the naked eye, and controls should always be used.

To show that the material is working correctly it is important to use the correct controls. If group A blood is to have its sub-type determined A_1 and A_2 should be used as controls, whilst for testing AB blood the controls should be A_1B and A_2B. Unless the anti-A_1 serum has been very recently tested with the controls so as to prove that it is working correctly, these controls must not be omitted, since the serum is always liable to change its properties on storage.

An anti-A_1 which gives specific results on slides may not necessarily do so in tubes, and one which works in tubes may fail to work on slides. The reagent must always be used by the method for which it is intended.

The cells of infants do not usually give satisfactory results with anti-A_1 owing to the incomplete development of the A antigen at birth.

Anti-O and Anti-H

ANTI-O AND ANTI-H AGGLUTININS (see Grubb, 1949)

These agglutinins were originally known as α_2; subsequently as anti-O, and are now divided into two classes known as anti-O and anti-H (Morgan and Watkins, 1948). Examples occur in the sera of oxen, eels and a variety of other animals, and some potent examples have been found in the sera of goats immunized with *B. dysentericae* Shiga. These two agglutinins are also of occasional natural occurrence in the sera of human beings. They may occur in the sera of group A_1, B or A_1B persons, and, very rarely, in the sera of group O persons (the "Bombay" bloods, see p. 125). Amongst twenty-five samples of anti-O and anti-H agglutinins which we studied, twenty-one came from A_1, three from B and one from an A_1B individual.

The exact nature of these sera has long been in doubt. It was originally thought that they were true anti-O sera reacting with the

product of the O gene. Later it was said that they reacted with the product of the A_2 gene also, but it now seems that this is not their true nature. An anti-O serum which we studied, for example, gave results of very different strength with different samples of group O blood, and when tested with group B bloods it gave many more negative results than would be expected if it were only failing to react with homozygous BB bloods, so that it could not be behaving as a true anti-O serum as defined above.

Anti-H sera are thought to react with an antigen H present on the red cell surface. The amount of H substance varies from one individual to another depending upon the ABO group. On the whole, there is least H present in A_1B and most in O bloods, and the amount present depends upon the following series:

$$A_1B < A_1 < A_2B < B < A_2 < O$$

This series shows the average amount of H substance present in bloods of each group if, say, a hundred examples of each group were tested, but there is a considerable "scatter" in the amount of substance present in the cells of different individuals of the same ABO group, so that, for example, an individual B blood may have less H substance than an individual A_1 blood (this probably depends partly on the genotype). Different anti-H sera vary in strength so that the proportion of positive results which they give within each ABO group varies from serum to serum. The weaker sera will only agglutinate cells carrying a lot of H substance. The result of this is that whilst different examples of anti-H serum give different percentages of positive results, the percentages within each ABO group will always be in the order shown above.

It is not apparent that anti-H and anti-O differ greatly from each other in their reactions with cells of various ABO groups and the distinction between these two sera depends on the fact that anti-H is neutralized by the H substance present in secretor saliva, whereas anti-O is not (Morgan and Watkins, 1948). O substance is not secreted in the saliva though it is sometimes weakly present in certain ovarian cyst fluids. The fact that anti-H can be neutralized by secretor saliva and anti-O cannot is correlated with the fact that individuals producing anti-H are usually non-secretors and those producing anti-O, secretors (Sanger, 1952). This in turn is correlated with the Lewis groups (see p. 127).

These antibodies appear to be of natural occurrence in human sera; [illegible] they act best at low temper- [illegible] and we have not known them to give rise to transfusion reactions or haemolytic disease, though the unusual examples of anti-H in group O individuals are perhaps rather different from the anti-H sera normally encountered. Anti-H and anti-O sera active only against papainized cells are sometimes seen.

In three cases which we studied, anti-H and anti-O agglutinins failed to pass through the placenta, though in a fourth case (p. 202) where a strong incomplete anti-H was present in the maternal serum it could be detected in the cord blood also, but did not appear to have any deleterious effect on the child. Some of the reactions of an unusually potent anti-H agglutinin which we encountered are shown in Table 37. It is interesting to notice that it contained a strong incomplete component active at 37° C. and demonstrable with anti-non-γ-globulin reagents.

TABLE 37. REACTIONS OF A POTENT ANTI-H SERUM

Cells	*Saline agglutinations* 16° C.	37° C.	*Coombs** *test at 37° C.*
O	v	++	++++
A_1B	++	—	—
A_1B (own cells)	—	—	—

* Reaction of anti-non-γ-globulin type.

The identification of [illegible] depends on showing that the s[illegible] positive results with cells of diff[illegible] groups. If an unknown antibody is encountered it should be tested in the first instance against a series of [illegible] against ten or twenty group O bloods and an equal number of group A bloods. The A bloods should be simultaneously tested with anti-A_1. If the serum under investigation came from a group B person, group B blood would, of course, have to be used instead of group A. In doing this test it is most important that all the cell samples tested should be of the same age and collected in the same manner, since one is comparing one set of results against the other. If the antibody gives a higher percentage of positive results with group O than with group A_1, or group B cells, it can only be anti-O, anti-H, anti-Le^b or anti-Le^a+Le^b. The last two can be excluded if the patient is Le(a−b+), and also by the reactions of the serum with cells of known Lewis type. A strong positive reaction with group O, Le(a−b−) cells is often a valuable diagnostic point. In practice, however, it must be admitted that the distinction [illegible] might be exp[illegible] antibodies, a[illegible] which are known to contain Lewis antibodies, nevertheless give a weak agglutination with certain Le(a−b−) cells. These points are discussed further on p. 231. If anti-Le^b can be

excluded, the distinction between anti-O and anti-H depends on whether the serum can be neutralized by secretor saliva. In carrying out this test one has to be careful in the case of the weaker antibodies not to be misled by the diluting effect of the saliva, and a control test with non-secretor saliva is advisable. The following example illustrates these procedures.

1. First ABO grouping test.

Patient's cells		*Patient's serum*	
Anti-A	*Anti-B*	*A cells*	*B cells*
v	–	++	v

2. Repeat test.

Patient's cells			*Patient's serum*			*Patient's cells with patient's serum*
Anti-A	*Anti-B*	*AB serum*	*A cells*	*B cells*	*O cells*	
v	–	–	++	v	+++	–

3. Further test.

Patient's cells				*Patient's serum*				*Patient's cells with patient's serum*
Anti- A_1	*Anti-A*	*Anti-B*	*AB serum*	A_1 *cells*	A_2 *cells*	*B cells*	*O cells*	
v	v	–	–	+	++	v	+++	–

4. Patient's serum tested with standard Group O cells.

Standard cells		*Coombs test*	*Albumin 37° C.*	*Saline agglutinations*	
				37° C.	16° C.
R_1R_1	N	–	–	–	+++
R_2R_2	JB	–	–	–	++
r r	S	–	–	–	+++
r r	C	–	–	–	++
r r	H	–	–	–	++
Patient's cells		–	–	–	–

5. Patient's serum tested with group O and A cells in saline at 16° C.

Group O cells		*Group A cells*		
No.	*Patient's serum*	*No.*	*Anti-A_1*	*Patient's serum*
1	+++	1	v	−
2	++	2	−	++
3	++	3	−	++
4	+++	4	v	+
5	+++	5	v	+
6	+++	6	−	+++
7	+++	7	v	w
8	+++	8	−	+++
9	++	9	v	−
10	++	10	v	−

6. Patient's serum tested with group O cells of various Lewis types in saline at 16° C.

Group O cells		*Patient's serum*
No.	*Lewis type*	
1	Le(a−b−)	+++
2	Le(a−b−)	++
3	Le(a+b−)	++
4	Le(a+b−)	+++
5	Le(a−b+)	+++
6	Le(a−b+)	++
7	Le(a−b+)	+++

7. Test for inhibition with saliva.
   One volume patient's serum plus one volume secretor saliva plus 2 volumes group O cells = −
   One volume patient's serum plus one volume non-secretor saliva plus 2 volumes group O cells = +++

Conclusion: Anti-H.

THE "BOMBAY" BLOODS

The cells of almost all group O individuals react strongly with anti-H and the agglutinin, anti-H, is consequently not found in their sera. The rare exceptions to this rule constitute the "Bombay" bloods, so called f[illegible] countered, although t[illegible] the world also (Bhen[illegible], Bhatia, 1955; Bhatia *et al.*, 1955; Simmons and D'Sena, 1955; Parkin, 1956). The cells of these individuals

give negative reactions with anti-A, anti-B and anti-H. Their sera contain not only anti-A and anti-B, but a potent anti-H also. The latter is often of high titre and may even react at 37° C. The serum will thus react with all cells except those of the patient himself and of other individuals of the same type, so that the provision of compatible blood for such patients presents a difficult though rare problem. With one very doubtful exception, all the bloods of this type encountered have been Le(a+) and in a case which was studied by Levine *et al.* (1955) it was shown that the phenotype O_h (a symbol used to designate the "Bombay" bloods) was not due to a new allele at the ABO locus, but to the action of a recessive suppressor gene, x. In double-dose this suppressor gene had the effect of suppressing the action of the genes "B" and "secretor", so that the person concerned appeared to be group O and a non-secretor, although genetically B,Ss. The presence of the genes B and S in this individual's genetic constitution was shown by the family study. The suppressor gene, x, also appeared to have some effect on the Lewis type, but the exact nature of this effect was not fully elucidated. This suppressor gene appears to be similar in its effect to the suppressor gene sometimes responsible for A_4 and similar weak forms of the A antigen.

Incomplete Anti-H

Dacie (1950) described the existence in the serum of normal individuals of a cold incomplete antibody which was subsequently shown to be of anti-H specificity (Crawford *et al.*, 1953).

In order to demonstrate the existence of this antibody it is necessary to obtain a fresh sample of blood and separate the serum from the clot at 37° C. Ten volumes of the serum are then mixed with one volume of a 20% suspension of the patient's cells and the mixture incubated for an hour at 4° C. or better still at 0° C. The cells are then washed four times and tested with Coombs reagent, when they will usually be found to give a positive result. The presence of autoagglutinins sometimes interferes with the test, but these can be removed from the cells by washing in warm saline at 37° C. This does not remove the incomplete autoantibody. The anti-H specificity of the antibody is shown by its reactions with cells of different ABO groups. The strongest reactions are obtained with group O cells and the weakest with A_1B cells. Intermediate reactions are obtained with cells of other groups, as in the case of anti-H agglutinatins.

The strength of the incomplete anti-H present in the serum varies in different individuals and it is entirely absent in some. It tends to be strongest in the serum of A_1B persons and weakest in the serum of group O persons. The antibody is called anti-H rather than anti-O, since it can be neutralized by secretor saliva; there is, however, a lesser degree of neutralization by some non-secretor salivas. The presence

of complement is necessary for the action of this antibody so that heat inactivation of the serum prevents its effects, and anti-complementary [illegible] interfere with the action of [illegible] ized by incomplete anti-H react only with anti-non-γ-globulin Coombs reagents (p. 61).

Transfusions of dextran may prevent the activity of this antibody in the recipient's serum; though the activity returns later. In one case after transfusion of dextran, activity of anti-H returned to the serum, but on this occasion the antibody activity in the serum was not prevented by the addition of dextran as it was before transfusion.

Table 38 shows the concentrations of clinical dextran and glucose required to prevent the activity of cold incomplete anti-H in 90 per cent of normal sera.

TABLE 38. THE EFFECT ON THE ACTIVITY OF COLD INCOMPLETE ANTI-H ANTIBODY OF THE ADDITION TO THE SERUM OF VARIOUS SUBSTANCES

Treatment of serum	*Anti-H activity*
1. Addition of Clinical Dextran 6 mgm./ml. ..	Negative
2. Addition of Glucose 5 mgm./ml.	Negative
3. Addition of Polyvinylpyrrolidone 10 mgm./ml.	Positive
4. Addition of 10% veronal buffered saline ..	Positive

Group Specific Substances

Seventy-eight per cent of individuals are known as secretors of the AB and H substances, and in these individuals it is possible to detect the substances in the saliva. The substances are present, although in lower concentrations, in other secretions. The ability to secrete group specific substances is a simple Mendelian dominant character determined by the presence of the gene S (secretor), and the character "Non-secretor" consequently behaves in a recessive manner and is determined by the allelic gene s. The gene S is very closely linked to the gene Le^b, and the gene s very closely linked to the gene Le^a. Since in adults the gene Le^b acts as a dominant gene and the gene Le^a acts as a recessive, it follows that Le(b+) persons are secretors of the ABH system, whilst Le(a+) persons are non-secretors.

Those who are phenotype Le(a−b−) may be secretors or non-secretors depending on their genetic constitution. The secretion of Lewis substances is independent of the genes S and s, which control the secretion of the AB and H substances only.

TECHNIQUES FOR DEMONSTRATING GROUP SPECIFIC SUBSTANCES

The A and B substances are usually easy to demonstrate in the saliva of secretors. The first step is to obtain a sample of saliva by asking the

individual concerned to spit into a wide test tube. Samples from infants may be obtained by swabbing out the mouth with cotton wool and squeezing the cotton wool into the tube. The tubes of saliva should be immersed in boiling water for ten minutes as soon as possible after collection in order to destroy enzymes which would otherwise inactivate the group specific substances. The saliva is then centrifuged at high speed to remove mucus and debris.

Inhibition test with anti-A and anti-B are best carried out on microscope slides at room temperature.

TECHNIQUE FOR INHIBITION TESTS. Mix 1 volume of anti-A or anti-B serum with 1 volume of saliva in a tube, wait for ten minutes for the reaction to take place, and then add 1 volume of a 5 per cent suspension of A or B cells, as appropriate, to 1 volume of the mixture on a microscope slide. The tests are mixed, rocked for six minutes and then read with the naked eye, and microscopically.

It should be remembered that the test is of a quantitative nature and suitable controls are very necessary. These should consist of known secretor and non-secretor salivas and of saline. It is also important not to have the anti-A and anti-B too strong, or low concentrations of the substances may be overlooked. This simple method will often suffice to show whether a person is a secretor or a non-secretor, provided that the controls are satisfactory. The control with saline should give a strongly positive result, and the control with non-secretor saliva should be almost as strong, whilst the control with secretor saliva should be negative or almost so. The speed with which agglutination occurs should be taken into account in assessing the results, as well as the final strength of the agglutination.

If clear-cut results with satisfactory controls are not obtained by this method, more extensive testing will be necessary, and a number of additional factors should be borne in mind. The amount of group specific substance needed to neutralize the agglutinin in a serum varies very widely from one serum to another. This is, of course, to some extent dependent on the titre, but by no means wholly so. This effect is illustrated by Table 39 and means that different sera will make the test vary in sensitivity.

One obvious way to increase the sensitivity of the test is to dilute the serum; indeed one can carry out the test in the form of a titration in which a volume of saliva is added to each of a series of dilutions of the serum. This makes the test quantitative and enables one to demonstrate minor degrees of inhibition. An example of this is shown in Table 40, where various salivas have been added to a series of dilutions of anti-B, prior to the addition of B cells. It can be seen in this table that even the O saliva (which contains no B substance) has a trace of inhibitory effect. This is presumably due merely to its viscous character which interferes

TABLE 39. VARIATION IN THE EASE OF NEUTRALIZATION OF ANTI-A BY A SALIVA

Name of serum	*Titre of serum*	*Highest dilution of saliva able to neutralize an equal volume of undiluted serum*
H 14	1 in 64	2 volumes undiluted saliva needed
Ar	1 in 64	1 in 2
Si	1 in16	1 in 256
3296	1 in 8	1 in 32
2404	1 in 8	1 in 1,000
Wr	1 in 4	1 in 16

to a slight extent with the formation of agglutinates. The B non-secretor has a somewhat greater inhibitory effect (perhaps it contains a trace of B substance) whilst the B secretor saliva has a marked effect. If the inhibitory effect of salivas varied in a continuous manner between these extremes one would have to draw a purely arbitrary distinction between secretors and non-secretors. This, however, is not the case, and there is almost always a clear-cut distinction between the powerful inhibitory effect of a secretor saliva and the relatively slight inhibitory effect of a non-secretor saliva.

TABLE 40. INHIBITION OF ANTI-B BY VARIOUS SALIVAS

Saliva	*Titre of anti-B serum*							
	1	2	4	8	16	32	64	128
B secretor	—	—	—	—	—	—	—	—
B non-secretor	v	v	++	+	—	—	—	—
O secretor	v	v	v	+++	++	—	—	—
Saline	v	v	v	v	+++	+	—	—

One volume of each dilution of the anti-B serum was mixed with 1 volume of undiluted saliva, and 2 volumes of cell suspension added.

Occasionally it is desirable to reduce the sensitivity of the test so as to avoid confusion due to minor degrees of inhibition and non-specific effects, and this may be done by adding a constant strength of serum to a series of dilutions of the saliva. However, this should not be done in an attempt to compensate for the use of an insufficiently strong serum in the first place.

Finally it may be mentioned that the reaction of anti-A with A_2 cells is more easily inhibited by A saliva than the reaction with A_1 cells. The former will, therefore, give a more sensitive test than the latter.

DEMONSTRATION OF H SUBSTANCE. The diagnosis of secretion in the case of group O individuals depends on the use of suitable anti-H serum. This may be of human or of animal origin and it may be necesary to do the test in tubes if the serum will not give satisfactory results on slides. Slides are to be preferred where possible, however, since by this method it is so much easier to compare one reaction with another. We ourselves use a selected normal ox serum for this purpose, and this particular serum has given satisfactory results for many years. It contains antibodies reading with A and B red cells and a species specific antibody, in addition to anti-H, but these do not interfere with the efficacy of the test. The antibodies reacting with A and B red cells are avoided by confining the test to group O cells, and the only effect of the weak species specific agglutinin is that the serum still reacts to some extent even when secretor saliva has been added to it. This reaction, however, is only weak whilst the un-neutralized serum gives a strong reaction with group O cells, so that in practice the results are clear cut. Not all ox sera are so satisfactory, however, and it is necessary to select one containing a high concentration of anti-H combined with a low concentration of species agglutinin. The way to do this is to take a number of ox sera and test them with secretor and non-secretor salivas selecting the one which shows the best inhibition.

USES OF SECRETOR SALIVA

Secretor saliva is a convenient means of neutralizing anti-A and anti-B in anti-Rh and other typing sera (p. 74). It is collected and processed as described on p. 128 and it should be remembered in this connection that saliva is usually rather hypotonic. If more than 1 volume of saliva has to be added to 1 volume of serum the mixture may be so hypotonic as to lead to lysis, and in such cases the saliva should first be made isotonic. The addition of $\frac{1}{20}$ volume of a 20 per cent solution of sodium chloride is a convenient means of doing this. In the case of weak antisera the diluting effect caused by adding saliva to the serum may sometimes be undesirable; this may be overcome by suspending the cells to be tested in secretor saliva instead of in saline. If this is done it is essential to make the saliva isotonic.

Another method which is sometimes useful, particularly in cases where the anti-A or anti-B in the serum to be tested would require more than an equal volume of saliva to neutralize it, is to concentrate the group specific substance in the saliva. This may be done by making use of the fact that the substances are insoluble in acetone.

TECHNIQUE FOR CONCENTRATION OF A AND B SUBSTANCES. The saliva should first be centrifuged at a high speed to remove all debris and obtain as clear a fluid as possible. Five volumes of acetone are then added to one volume of the clear saliva and the resultant cloudy

mixture centrifuged at high speed to throw down a precipitate containing the group specific substances. The supernatant fluid is then removed and discarded and the acetone removed from the precipitate by warming the tube slightly and by applying a partial vacuum to the tube by means of a Bunsen water-suction pump. When the precipitate no longer smells of acetone it may be suspended in a few drops of saline, and it will be found that this product is highly inhibitory to anti-A or anti-B. It is best to prepare this product freshly when required, since we have the impression that the acetone treated substances tend to be unstable.

Immune Anti-A and Anti-B

Anti-A and, less commonly, anti-B sometimes show certain properties which are taken to indicate an immune response and to indicate that in certain circumstances the antibodies may be of more clinical significance than the anti-A or anti-B ordinarily encountered. These properties are found more frequently in the sera of group O persons than in those from group A and group B persons.

Occurrence

Immune anti-A and anti-B occur in the following circumstances:

1. In the serum of mothers of cases of haemolytic disease due to ABO incompatibility.
2. Following ABO incompatible transfusions.
3. Following the injection of antigens.
4. Without ascertainable cause.
5. In the mothers of normal ABO incompatible infants.

Immune characteristics may appear in the sera of persons who have been experimentally injected with A or B polysaccharides of human or animal origin for the production of potent anti-A and anti-B typing sera. They also occur following exposure to a number of antigens which have an A, or A-like, specificity. Horse serum is one of these, and therapeutic or prophylactic injections of serum prepared by immunizing horses against tetanus, diphtheria and the like, often give rise to these properties. T.A.B. vaccine also contains an A-like antigen derived from the peptone on which the [illegible] immune characteristics may [illegible] Cases of infectious mononu[illegible] also show these properties.

These properties are sometimes found in the sera of people who, so far as can be ascertained, have not been exposed to any of the [illegible] mentioned above. They [illegible] tion against bacterial an[illegible] of pneumococci, for example, are known to contain such antigens.

PROPERTIES

The properties which are understood to constitute the immune characteristics of an anti-A or anti-B serum are variable, and some of them are to be found in one serum and others in another, so that not all the immune sera are of the same type. They are properties indicative either of a high concentration of antibody or of the presence of incomplete antibody, and are as follows:

1. *High titre. A saline agglutinating titre greater than 1 in 512 can* be considered as an immune characteristic. In one group O patient, who was accidentally transfused with group A blood, the titre subsequently rose to 1 in 1,000,000.

2. *Neutralization of agglutinin by group specific substances.* The amount of A secretor saliva required to inactivate the agglutinin in anti-A sera varies very widely. With some sera the agglutinin can be completely neutralized even by saliva diluted one in a thousand, and at the other extreme sera are encountered which require as many as 8 volumes of undiluted saliva to neutralize their agglutinins. If a serum requires a large amount of A substance to neutralize the agglutinin this is taken as an immune characteristic. A somewhat arbitrary working rule is to consider as an immune serum any serum requiring more than an equal volume of undiluted saliva to neutralize the agglutinin.

3. *Enhancement of titre by serum.* If the serum gives a higher titre when the cells are suspended in serum and the dilutions made in serum, than it does when saline is used, this is taken to indicate the presence of an incomplete anti-A (or anti-B). This test suffers from the disadvantage that it is dependent upon the saline agglutinin titre and will, therefore, only give a positive result when the incomplete antibody has a higher titre than the agglutinin. Incomplete anti-A, like anti-Kell, is best potentiated by human serum. Bovine albumin has but little potentiating effect. It is difficult to know what group of serum to use as a diluent for this test; groups O and B sera contain anti-A agglutinins and groups A and AB sera contain A substance—in some cases at any rate. Group B serum from which the anti-A has been removed by absorption could be used, but is open to the theoretical objection that the absorptive process may liberate some A substance into the serum from the absorbing cells. A further consideration is that the enhancing effect varies from one serum to another. On the whole we are inclined to think that these factors are not really of great importance, and in practice it is convenient to use A or AB serum as a diluent.

4. *Antiglobulin tests.* The incomplete antibody in these sera is often less easily neutralized by A substance than is the agglutinin. In the case of anti-A this may perhaps be correlated with the fact that incomplete anti-A acts to a large extent as an anti-A_1. It will be recalled

that anti-A_1 is more difficult to neutralize than anti-A. The fact that incomplete anti-A acts predominantly as an anti-A_1 means that stronger results are obtained in all the tests with A_1 than with A_2 cells, and the fact that it is more difficult to neutralize by saliva than the agglutinin enables one to neutralize the agglutinin and then carry out an anti-globulin test for incomplete antibody.

Both anti-γ-globulin and anti-non-γ-globulin should be used in the test, since the antibody may be of either type. Many incomplete anti-A sera do not react well with either reagent, however, so that this test is often insensitive.

5. *Zoning.* This is only encountered very rarely, but we have encountered it once following an ABO incompatible transfusion.

6. *Thermal amplitude.* Anti-A and anti-B usually have a higher titre at 16° C. than at 37° C. If the titre is the same at the two temperatures and more especially if it is higher at 37° C. than at 16° C. this is taken as an immune characteristic.

7. *Haemolysis.* Specific lysis of A_1 and less frequently of A_2 and B cells, is often seen; the lysis is dependent upon the presence of complement so that it is only seen with fresh sera, and the haemolysin is easily neutralized by small quantities of group specific substances. The necessity for complement introduces a second variable into the test which, to some extent, reduces its value. It also makes it difficult to titrate the haemolysin (p. 55).

8. *Agglutination of serum suspended cells by partially neutralized serum.* After the agglutinin has been neutralized by A secretor saliva the mixture of serum and saliva is titrated against A_1 cells suspended in compatible human serum, using the same serum as diluent in the titrations. The occurrence of agglutination is taken as an immune characteristic.

(See: Witebsky, 1948; Mollison, 1956.)

Methods of Detection

The method which we use to investigate anti-A and anti-B sera for the presence of immune characteristics is as follows. It is designed to reveal any of the characteristics mentioned above if they are present.

1. Titrate the serum against A_1 (or B) cells in saline and also in serum at 16° C. and at 37° C. Use fresh serum of a group compatible with the cells and include control tests of cells suspended in the fresh serum diluent, in order to exclude any agglutinating activity by the diluent. Inspect the tubes for haemolysis before reading the tests.

2. Mix the serum with an equal volume of A (or B) secretor saliva, stand for ten minutes at room temperature and carry out the following tests:

(*a*) Titrate the mixture at 37° C. against A_1 (or B) cells suspended in saline and in serum, using the serum as the diluent in the titration

in the second case. The diluent must be controlled as in (1) unless it is the same serum.

(*b*) Carry out an indirect Coombs test at 37° C. using A_1 (or B) cells. Test the washed cells with anti-γ-globulin reagent, anti-non-γ-globulin reagent, and saline. The purpose of the saline control is to see whether the agglutinin has been neutralized, and it is necessary to do it in this manner because a weak agglutination due to small amounts of unneutralized agglutinin may break up during the washing process but re-form when the cells are rocked on a microscope slide.

The application of these methods may be illustrated by the following case:

CASE 30

Mrs. St., a primigravida, had never had a transfusion, but had always been subject to slight hay fever. She received two injections of anti-tetanus serum, ten years and three weeks before the birth of her infant. She was delivered of a full-term male child, which was jaundiced at birth and had a haemoglobin of 10·5 gm. per cent and a serum bilirubin of 8 mg. per cent. The infant died when three days old. Serological investigations were as follows:

Mother: Group OR_1R_2
Father: Group A_1R_1r
Infant: Group AR_1r direct Coombs test negative.

No antibodies were detected when the mother's serum was tested by the Coombs test, papain cell test, albumin and saline agglutinations at 16° C., and 37° C. with a series of standard cells.

Test for immune anti-A.

Method 1. SIMPLE TITRATIONS AGAINST A_1 CELLS

Temp. °C.	*Medium*	*Titre of serum*												
		1	2	4	8	16	32	64	128	256	512	1,000	2,000	*Control*
16	Saline	h	h	v	v	v	v	+++	+	w	—	—	—	—
16	Serum	h	v	v	v	v	v	v	+++	++	+	—	—	—
37	Saline	h	h	h	+++	+++	+++	++	w	—	—	—	—	—
37	Serum	h	h	v	v	v	v	v	+++	++	++	++	—	—

h = haemolysed.

Method 2a. AGGLUTINATION OF SERUM SUSPENDED A_1 CELLS BY PARTIALLY NEUTRALIZED SERUM

Temp. °C.	*Medium*	*Titre of serum and saliva mixture*									
		1	2	4	8	16	32	64	128	256	*Control*
16	Saline	—	—	—	—	—	—	—	—	—	—
16	Serum	v	v	+++	++	++	+	w	—	—	—
37	Saline	—	—	—	—	—	—	—	—	—	—
37	Serum	v	v	+++	+++	+++	++	++	+	—	—

Method 2b. INDIRECT COOMBS TEST WITH SALIVA

Anti-γ-globulin reagent	+++
Anti-non-γ-globulin reagent ..	—
Saline control	—

Remarks: As is usual, this serum shows some, but not all, of the immune characteristics. The properties of thermal amplitude, haemolysis, enhancement of titre by serum, positive indirect Coombs test, and agglutination of serum suspended cells by partially neutralized serum were seen, but the titre was not unusually high and the agglutinin was not difficult to neutralize by secretor saliva. These properties, combined with the clinical history and the absence of any antibodies of other specificities, led to the diagnosis of haemolytic disease due to the presence of immune anti-A.

DANGEROUS UNIVERSAL DONORS

When group A patients are transfused with group O blood, some destruction of the recipient's cells due to the action of the anti-A in the donor's blood is occasionally seen, and when this happens it is almost always found that the anti-A in the donor's blood shows immune characteristics. The same thing could presumably happen to a group B patient, though this seems to be uncommon. The occasional occurrence of these cases has given rise to the concept of the "Dangerous Universal Donor" whose anti-A or anti-B shows immune characteristics and whose blood is therefore considered unsuitable for transfusion to patients of groups other than O. Various tests have been designed for the detection of such donors, but this is a difficult matter owing to the fact that different immune anti-A and anti-B sera show the various properties enumerated above in varying degrees, and no single property is a constant feature of all such sera. Furthermore, it is not known whether all the properties are equally dangerous and it is very difficult to obtain any firm correlation between these properties and the clinical findings.

HAEMOLYTIC DISEASE DUE TO ANTI-A AND ANTI-B

Cases of haemolytic disease due to anti-A and anti-B are not infrequent, but, unfortunately, the diagnosis of this condition does not rest on the same secure grounds as the diagnosis of haemolytic disease due to other blood group antibodies. This is due to the variable character of the immune characteristics shown by different anti-A and anti-B sera and to the fact that these properties can be found even in the serum of group O mothers of apparently healthy group A children.

The diagnosis thus becomes difficult to make and can only be arrived at by taking into account all the relative clinical and serological features of the case. These cases often occur in the first-born child of the family and occasionally the mother gives a history of immunization by A.T.S., T.A.B. and the like, sometimes followed by allergic manifestations. Moreover, as Rosenfield (1955) has stated, the mother is almost always group O and group A children are more likely to be affected than group B children. Jaundice is a more marked, and anaemia a less marked feature than in the case of haemolytic disease due to Rh incompatibility.

The diagnosis of haemolytic disease due to ABO incompatibility should not be made unless the clinical and haematological condition of the child is suggestive of the disease (see Mollison, 1956) and the mother's serum shows evidence of immune anti-A or anti-B and an absence of antibodies of other specificities. The mother's serum ought to be tested for as many other blood group antibodies as practicable. Tests of the mother's serum against the husband's cells are not usually helpful since they are complicated by ABO incompatibility. One can, of course, carry out an indirect Coombs Test using the husband's cells after the agglutinin in the maternal serum has been neutralized by secretor saliva and if this is positive it may be due either to incomplete anti-A or anti-B in the mother's serum or to some other antibody in the mother's serum reacting with the husband's cells. The next step in such a case would be to repeat the test using the husband's cells and other A_1 cells as well, but even if this gives positive results with the husband's cells alone, this does not necessarily mean that it is not due to incomplete anti-A (p. 205).

Absorption with other A cells would, of course, settle the matter, but unless the mother's serum shows a marked difference in reaction between the father's and other A cells, and fails to show ABO immune characteristics, it hardly seems necessary to pursue the matter as far as this.

The cord cells in these cases often exhibit spherocytosis together with increased fragility. Sometimes, free anti-A can be detected in the cord serum of affected group A children by using the papain or antiglobulin

test, and sometimes the unwashed cord cells will agglutinate when suspended in adult serum on a slide at 37° C. (Witebsky *et al.*, 1947), although neither of these tests is invariably positive. The result of the direct Coombs Test on the cord blood will depend upon the manner in which it is performed. Using the technique which we describe it is often entirely negative and even when it is positive the reaction is weak and only persists for the first few days of the child's life. Rosenfield (1955), using his special antiglobulin technique found a direct positive test in 11 per cent of group incompatible infants but none in compatible ones. He considered that this antiglobulin test detected an abnormal class of group incompatible infants whose mean haemoglobin is low and whose mean reticulocyte count and bilirubin are high when compared with those of other group incompatible infants. He also found that 38 of 39 mothers of incompatible infants with positive direct antiglobulin tests were group O; this is a significant disproportion.

References

Beckers, T., Loghem, J. J. van, and Dunsford, I. (1955). A second example of the weak antigen A_4 occurring in the offspring of group O parents. *Vox Sang.*, 5, 145.

Bell, R. (1953). The recognition of a rare blood (A_4) in a clinical laboratory. *J. Med. Lab. Tech.*, 11, 185.

Bhatia, H. M. (1955). ABO blood groups and the H-substance. Editorial: *J. Ind. Med. Assoc.*, 25.

Bhatia, H. M., Sanghvi, L. D., Bhide, Y. G., and Jhala. H. I. [illegible]

Bher[illegible] blood group character related to the ABO system. *Lancet*, i, 903.

Cahan, A., Jack, J. A., Scudder, J., Sargent, Mary, Sanger, Ruth, and Race, R. R. (1957). A family in which A_x is transmitted through a person of the blood group A_2B. *Vox Sang.*, 2, 8.

Crawford, H. Cutbush, Marie, and Mollison, P. L. (1953). Specificity of incomplete "cold" antibody in human sera. *Lancet*, i, 566.

Dacie, J. V. (1950). Occurrence in normal human sera of "incomplete" forms of "cold" auto-antibodies. *Nature*, 166, 36.

Dacie, J. V. (1954). *The Haemolytic Anaemias*, pp. 261, 258. London: J. and A. Churchill Ltd.

Dunsford, I. (1952). Group A blood weaker in reaction than A_2. *Bull. of Cent. Lab. Neth. Red Cross Blood Trans. Ser.*, 2, 209.

Dunsford, I. (1955). A_4 versus factor C. *Vox Sang.*, 5, 104.

Dunsford, I., and Aspinall, P. (1952). An A_4 cD^ue blood in an English family. *Ann. Eugen.*, 17, 30.

Dunsford, I., Stacey, S. M., and Yokoyama, M. (1956). A rare variety of the human blood group B. *Nature*, 178, 1167.

Ellis, F. R., and Cawley, L. P. (1957). A third report of agglutinogen A_0. Occurrence in mother and child. *Amer. J. Clin. Path.*, 27, 438.

Haemolytic Disease due to Anti-A and Anti-B

Cases of haemolytic disease due to anti-A and anti-B are not infrequent, but, unfortunately, the diagnosis of this condition does not rest on the same secure grounds as the diagnosis of haemolytic disease due to other blood group antibodies. This is due to the variable character of the immune characteristics shown by different anti-A and anti-B sera and to the fact that these properties can be found even in the serum of group O mothers of apparently healthy group A children.

The diagnosis thus becomes difficult to make and can only be arrived at by taking into account all the relative clinical and serological features of the case. These cases often occur in the first-born child of the family and occasionally the mother gives a history of immunization by A.T.S., T.A.B. and the like, sometimes followed by allergic manifestations. Moreover, as Rosenfield (1955) has stated, the mother is almost always group O and group A children are more likely to be affected than group B children. Jaundice is a more marked, and anaemia a less marked feature than in the case of haemolytic disease due to Rh incompatibility.

The diagnosis of haemolytic disease due to ABO incompatibility should not be made unless the clinical and haematological condition of the child is suggestive of the disease (see Mollison, 1956) and the mother's serum shows evidence of immune anti-A or anti-B and an absence of antibodies of other specificities. The mother's serum ought to be tested for as many other blood group antibodies as practicable. Tests of the mother's serum against the husband's cells are not usually helpful since they are complicated by ABO incompatibility. One can, of course, carry out an indirect Coombs Test using the husband's cells after the agglutinin in the maternal serum has been neutralized by secretor saliva and if this is positive it may be due either to incomplete anti-A or anti-B in the mother's serum or to some other antibody in the mother's serum reacting with the husband's cells. The next step in such a case would be to repeat the test using the husband's cells and other A_1 cells as well, but even if this gives positive results with the husband's cells alone, this does not necessarily mean that it is not due to incomplete anti-A (p. 205).

Absorption with other A cells would, of course, settle the matter, but unless the mother's serum shows a marked difference in reaction between the father's and other A cells, and fails to show ABO immune characteristics, it hardly seems necessary to pursue the matter as far as this.

The cord cells in these cases often exhibit spherocytosis together with increased fragility. Sometimes, free anti-A can be detected in the cord serum of affected group A children by using the papain or antiglobulin

Weiner, W., Lewis, H. B. M., Moores, Phyllis, Sanger, Ruth, and Race, R. R. (1957). A gene y, modifying the blood group antigen A. *Vox Sang.*, 2, 25.

Wiener, A. S. (1948). *Blood Groups and Transfusion. 3rd Ed.* Illinois: Charles C. Thomas.

Wiener, A. S. (1953). The blood factor C of the ABO system, with special reference to the rare blood group C. *Ann. Eugen.*, 18, 1.

[illegible] hitherto undescribed human [illegible] the Rh system and the AB system. *Blood*, 3, 66.

Witebsky, E., Mitchell I. Rubin, Engasser, Lillian M., and Blum, Livia (1947). Studies in erythroblastosis foetalis. II. Investigation on detection of sensitization of red blood cells of newborn infants with erythroblastosis foetalis. *J. Lab. and Clin. Med.*, 32, 1339.

Engelfried, J. J. (1955). Another instance of blood subgroup A_0. *Amer. J. Clin. Path.*, **22**, 1157.

Estola, E., and Elo, J. (1952). Occurrence of an exceedingly weak "A" blood group property in a family. *Ann. Eugen.*, **17**, 30.

Fischer, W., and Hahn, F. (1935). Ueber auffallende Schwache der gruppenspezifischen Reaktionsfahigkeit bei eineim Erwachsenen. *Z. Immun. Forsch.*, **84**, 177.

Friedenreich, V. (1936). Eine bisher unbekannte Blutgruppeneigenschaft (A_3). *Z. Immun. Forsch.*, **89**, 409.

Gammelgaard, A. (1942). *Om Sjaeldne, Svage A—receptorer (A_3, A_4, A_5, og A_x) Hos Mennesket.* Published by *Nyt Nordisk Forlag.* Arnold Busck, Copenhagen (p. 137).

Gammelgaard, A., and Marcussen, P. V. (1940). Nachweis eines vierten allelomorphen A—gens (A_4). *Z. Immun. Forsch.*, **98**, 411.

Grove-Rasmussen, M., Soutter, L., and Levine, P. (1952). A new blood subgroup (A_0) identifiable with group O serums. *Amer. J. Clin. Path.*, **22**, 1157.

Grubb, R. (1949). Some aspects of the complexity of the human ABO blood groups. *Acta Path. Microbiol. Scand.*, Suppl. 84, 1–72.

Levine, P., Robinson, E., Celano, M., Briggs, Olive, and Falkinburg, L. (1955). Gene interaction resulting in the suppression of blood group substance B. *Blood*, **10**, 1100.

Levine, P., Celano, M., Griset, T. (1956). B_w, a genetic variant of blood factor B. *VIth Cong. of Inter. Soc. of Blood Trans.*, p. 132.

Loghem, J. J. van, and Hart, Mia van der (1954). The weak antigen A_4 occurring in the offspring of group O parents. *Vox Sang.*, **4**, 69.

Loghem, J. J. van, Dorfmeier, Hanny, and Hart, Mia van der (1957). Two A antigens with abnormal serological properties. *Vox Sang.*, **2**, 16.

Mollison, P. L. (1956). *Blood Transfusion in Clinical Medicine.* 2nd Ed. Oxford: Blackwell Scientific Publications.

Morgan, W. T. J., and Watkins, Winifred M. (1948). The detection of a product of the blood group O gene, and the relationship of the so-called O substance to the agglutinogens A and B. *Brit. J. Exp. Path.*, **29**, 159.

Moullec, J., Sutton, E., Burgada, M. (1955). Une variante faible de l'agglutinogène du groupe B. *Rev. d'Hém.*, **10**, 574.

Parkin, Dorothy (1956). *Study of a family with unusual ABO phenotypes. Brit. J. Haem.*, **2**, 106.

Race, R. R. (1957). Editorial: *Vox Sang.*, **2**, 2.

Rosenfield, R. E. (1955). A-B Hemolytic Disease of the Newborn. Analysis of 1,480 cord blood specimens with special reference to the Direct Antiglobulin test and group O mother. *Blood*, **10**, 17.

Sanger, Ruth (1952). A relationship between the secretion of the blood group antigens and the presence of anti-O or anti-H in human serum. *Nature*, **170**, 78.

Simmons, R. T., and D'Sena, G. W. L. (1955). Anti-H in group O blood. *J. Ind. Med. Assoc.*, **24**, 325.

Stratton, F., Renton, P. H., and Hancock, Jeanne A. (1958). Red cell agglutinability affected by disease. *Nature*, **181**, 62.

that this checking process in some way absolves them from care in the first part of the process, i.e. in placing the tubes in the rack. They assume that they need not worry about an occasional mistake when placing the tubes in the rack, since this will be detected in the checking process. Such an attitude is, of course, quite wrong and largely invalidates the efficacy of the check. Such people fail to realize that the check is, itself, subject to error in the same way as the original process, and they do not understand the principles involved. The accuracy required in ABO grouping is so great that each step must be carried out as accurately as possible, and each step must be independently checked as well.

It is also very desirable to exercise a statistical control of the whole process by observing how many errors at each stage are detected by the checking process and computing from this the probable number of cases in which a mistake in the first place will coincide with a mistake in the checking process so that an error will be completely overlooked. It is extraordinarily difficult to get those concerned in these tests to appreciate these points, particularly the more junior workers. It is therefore essential to have a rigid and detailed routine whereby each step is written down and clearly defined, and to impress on the more junior workers that they must on no account deviate in the slightest degree from this routine. In addition, a more senior person, thoroughly conversant with the routine and with the principles involved in it, should be in continuous and detailed supervision of the more junior workers in order to see that they adhere closely to the routine and that they are not so careless at any stage in the process as to invalidate the checking procedure.

The details of the actual routine employed will naturally vary from one laboratory to another according to local circumstances, but the important thing is the principle on which the routine is founded. In order to illustrate the way in which these principles may be put into operation, we propose to describe in some detail the ABO grouping procedure in our own laboratory, and then to discuss the way in which these principles are exemplified in this particular routine. We do not, of course, suggest that our particular routine is necessarily the one which should be rigidly adhered to by other laboratories.

Routine for Large Scale ABO Grouping

The routine which we employ rests on the following points:

1. The use of the tube technique; reading by tapping.
2. Tests of the donors' cells with anti-A and anti-B.
3. Tests of the donors' sera with A_1 and B cells.
4. Tests of the donors' cells with group O serum as an additional check.

CHAPTER VII

THE ORGANIZATION OF LARGE-SCALE ABO GROUPING

The Nature of the Problem

THE correct determination of the ABO groups of half a dozen samples of blood is one of the easiest things in the whole field of blood group serology, but the correct determination of the ABO groups of, say, 100,000 samples of blood is one of the most difficult, and there must be few, if any, laboratories engaged in large-scale ABO grouping who have not been guilty of errors at one time or another. The reasons for this paradox, and the true nature of the difficulties involved, are not always appreciated even by those who have considerable experience of ABO grouping.

The primary reason for these difficulties is the very high standard of accuracy required in this test due, of course, to the grave consequences which can so easily follow from incorrect ABO grouping. To take a concrete example, our own laboratories are at the present time determining the ABO groups of rather more than 60,000 blood donors per annum, and if we were to send out one incorrectly grouped bottle of blood each year we would certainly not consider that a satisfactory standard had been reached. In other words, we require less than one mistake in 60,000 tests. Now an accuracy of this order is not easy to obtain and it is doubtful whether even the most experienced worker could carry out any single test (or even any single manipulation) 60,000 times without making a single mistake, and the only way in which the required standard of accuracy can be reached is by multiple tests and by checking each step in the procedure. Let us suppose a given worker will make one mistake in a thousand tests. If each test is carried out twice, taking care that the first test and the repeat test are entirely independent of one another, only one mistake in a million tests should then result. This is the basic principle of checking on which success in large-scale ABO grouping depends, and it is important to have a clear understanding of the mathematical basis on which its efficacy rests. The nature of the checking process is often misunderstood, and it is not always realized that the check will only be fully effective if both the test and the check are entirely independent of one another and if each has, itself, a good standard of accuracy. For example, if numbered tubes are to be placed in a rack in numerical order, the process should be checked by examining the numbers on the tubes a second time after they have been placed in the rack, when it will sometimes be found that a mistake was made when the tubes were originally placed in the rack. Inexperienced workers frequently assume

NBTS REGIONAL CENTRE, ROBY STREET, MANCHESTER 1

BLOOD GROUP RECORD

Clerk	Place	Team	Date
A N OTHER	ROBY STREET	X	12 · 10 57

No.	Name (capitals)		Kahn Number	Group: Sorted	Group: Check	Group: Rh	Remarks
1	JONES	MISS M S	BK 2731	A		+	
2	CROWN	MR D	2732				
3	THOMAS	MRS W	2733				
4	TAYLOR	MR V	2734				
5	DEAN	MR M	2735	O		—	
6	BENNETT	MISS M	2736	A		+	HIGH TITRE
7	HARDY	MRS A	2737				
8	FANNELL	MR E	2738				
9	JAMES	MISS K	2739				
10	GRUNDY	MISS L	2740				
11	SIDEBOTHAM	MR J	2741				
12	HOGG	MR W	2742	O		R′	
13	KONNICK	MR A	2743				
14	DEWY	MISS E	2744				
15	SHELMERDINE	MISS C	2745	B		+	
16	WAGSTAFF	MR S	2746				
17	TRAYNOR	MR F	2747				
18	MURPHY	MISS J	2748				
19	McINTYRE	MR P.	2749				
20	HOWES	MR D	2750				
21	BROAD	MISS J	2751	A		+	
22	EDDLESTON	MR P	2752	A		+	
23	HEMSLEY	MR R	2753	O		+	
24	MOSER	MR E.	2754				
25	THOMPSON	MISS M	2755	O		—	
26	ARTHUR	MR C	2756				
27	WOOD	MR F	2757				
28	HOLLAND	MISS B	2758				
29	~~CHADWICK~~	~~MRS H~~	~~2759~~	~~AB~~		~~+~~	~~FEW CC~~
30	SUMMERS	MR D	2760				
31	WRIGHT	MR H	2761				
32	WALL	MISS V.	2762				
33	WESTON	MR S.	2763	B		R″	
34	BOOTH	MRS P	2764				

FIG. 2. Blood collection sheet.

5. Repeat tests where discrepancies occur, carried out by the tube technique, reading microscopically.
6. The use of specially designed equipment intended to reduce the occurrence of mistakes to a minimum. This comprises:
   (*a*) A printed blood collection sheet for recording the donors' names at the blood collection session (Fig. 2).
   (*b*) A printed laboratory work sheet (Fig. 3).
   (*c*) Printed, serially numbered labels for the blood bottles and pilot samples.
   (*d*) A set of specially made racks for housing the tubes and carrying out the tests in an orderly fashion.
7. The use of a detailed procedure by which the tests are carried out, so designed that each step is subjected to at least one independent check.
8. Detailed supervision of the work to maintain rigid adherence to the procedure.
9. A statistical control of the whole process in order to ascertain whether the standard of work is of a satisfactory degree of safety.

The process of ABO grouping does not merely consist of testing some sera against some cells, it is a process which starts when a donor walks in to the blood collection session, and which is not complete until a label showing the correct group has been stuck on to the bottle of blood. We are firmly of the opinion that reliance ought not to be placed on donors' statements of their groups, or on previous records of their groups, but that the donor's group ought to be determined each time he attends, and that no bottle of blood ought to be labelled with a group label until the blood donated on that occasion has been tested. In other words, we determine the group of a bottle of blood rather than the group of a donor, though records of previous tests on the donor do, of course, constitute a valuable check of the tests carried out on a subsequent occasion.

Fig. 3 shows the laboratory work sheets. These are duplicate sheets interleaved with carbon paper. One copy is used to record the results of tests on the donors' cells and the other of tests on the donors' sera. The top sheet is narrower than the bottom one so that both sets of results can be seen together in contiguous columns when the top sheet is placed over the other. In this way the two tests are recorded independently of one another. An essential feature of this arrangement is that the two sheets can be separated from one another so that those who are reading and recording the results of tests on the donors' cells can have no prior knowledge of the results with the donors' sera. The reason for this is not to avoid deliberate falsification of the results (anyone who could do this would in any case be quite unfit to work in a blood grouping laboratory), but because prior knowledge of the

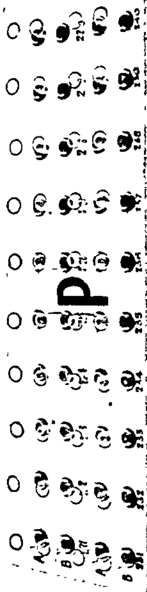


FIG. 3 Blood grouping Sheets.

results confers an unconscious bias by which it is almost impossible to avoid being influenced. Moreover those who are recording the results become so used to the four patterns of results given by the four groups that they tend to write them down instinctively. Another reason for being in ignorance of one half of the test while testing the other half is that if a discrepant result is called out the person recording the results might be tempted to express surprise, or doubt its accuracy, and this could lead the person reading the results to revise his opinion, perhaps incorrectly.

The specially constructed racks (Plate XIV) are made so that they fit on to one another by means of a peg and socket, and all the holes are serially numbered with paint. The number of holes in the rack is in multiples of ten so as to correspond with the number of spaces on the laboratory record sheet. Furthermore, the pegs and sockets are so arranged that all racks containing tests of the first twenty donors fit together, but none will fit on to the racks belonging to the second twenty donors, and so forth. A set of racks comprises:

1. A rack for pilot tubes of donors' blood.
2. A rack for tests of the donors' sera.
3. A rack for tests of the donors' cells.
4. A rack for separated donors' sera.

These four racks are concerned in the determination of the donor's ABO group, but the complete set of racks also includes:

5. A rack for the sera of group A donors to be tested for the selection of high titre grouping serum.
6. Ditto—for group B donors.
7. Rack for tests of the donors' Rh groups. This rack is used for putting up the tests.
8. Duplicate of 7 kept warm in a 37° C. incubator, and used for incubating the tests.
9. Rack for the serum of D negative donors for antibody testing.
10. A metal rack for the sera of donors for inactivation of the sera prior to carrying out the Kahn test.

The holes in all the racks are equally spaced so that there can be no doubt as to which hole in one rack corresponds to a given hole in another rack when the two are fitted together. There is also a double row of holes in rack 1 so that one can move the tubes from one row of holes to the other as the work proceeds, in order to keep one's place (see Plate XIV).

A description of the procedure which we use for large-scale ABO grouping follows and it will be noticed that the procedure incorporates checks at each stage.

the pilot tube, and, if this is done correctly, it will be found that a suitable cell suspension can be made on the addition of saline. If only one pilot tube is removed from the rack at a time and is replaced in its correct hole as soon as the cell suspension is prepared, it hardly seems likely that the order of the pilot tubes in the rack will be disarranged. The rack of tubes of separated serum is set aside and used for Kahn tests, antibody tests, repeat tests of group where necessary, etc. The use of the centrifuge at this stage should be avoided if possible, owing to the danger of disarranging the order of the tubes in the rack. If the serum is not free from cells, however, centrifugation will be necessary and a further check of the order of the tubes in the rack will have to be carried out.

8. **Test of donors' cells.** Three rows of $2 \times \frac{3}{8}$ inch tubes are placed in the appropriate rack. One vol. (0·08 ml.) of anti-A grouping serum is placed in each tube of the first row; 1 vol. of anti-B in the second row, and 1 vol. of group O serum in the third row. The tubes are then inspected to see that each has, in fact, received its quota of serum. Each $2 \times \frac{3}{8}$ inch tube then receives 1 vol. of cell suspension from one of the pilot tubes, working from left to right along the row, and observing all the precautions enumerated in item (6) above. The tubes are then tapped to mix their contents and left on the bench to sediment.

9. **Reading results with donors' sera.** After one and a half hours' incubation at room temperature the results are read. One person takes each tube in turn from its place in the rack, taps gently and reads the result, naked eye, whilst a second person records the result on the sheet. At the end of every tenth tube a check is made by noting that the tenth place on the laboratory work sheet has been reached. This is to avoid the common mistake of omitting to record a result so that all subsequent results are displaced upwards by one place. When the tests have been read the tubes must be discarded, so that there is no temptation to look at them again and revise the original reading if it is found that the results disagree with those for the donors' cells. In reading the results, clear-cut positive or negative results are not seen in every tube. When the result is in doubt, it is most important that it should be honestly recorded as "doubtful" so that the test can be repeated subsequently. The tendency to take the plunge in a doubtful case and to call it either "positive" or "negative" must be resisted.

10. **Reading results with donors' cells.** This is done in an exactly simil[illegible]
shee[illegible]
We regard this as an essential point.

Steps 6–10 constitute the serological determination of the ABO group, and the tests on the donors' cells are carried out independently of the tests on the donors' sera, so that one test checks the other.

11. **Special tests to exclude A_2B.** In reading results with donors'

DETAILED PROCEDURE FOR LARGE-SCALE ABO GROUPING

1. Delete from the blood collection sheet (Fig. 2) any bottles which are not to be tested, e.g. where vene-puncture failed. These bottles are included with the others at the blood collection session so as not to disturb the serial numbering.

2. Place the pilot tubes in order according to their serial numbers in the appropriate rack. This is done by two persons; one calling out the numbers from the blood collection sheet and the second placing the tubes in the rack in the corresponding order.

3. Check (2) by one person calling out the numbers of the tubes which are already in the rack whilst a second person checks the order by consulting the sheet.

Note that it is unsatisfactory for one person alone to attempt to do both these things, owing to the danger of losing one's place on the sheet.

4. Enter the serial numbers of the blood to be grouped from the blood collection sheet on to the laboratory work sheet.

5. Check the serial numbers on the laboratory work sheet against the serial numbers on the pilot tubes already in the rack. This is primarily to check that the numbers have been correctly inscribed on the work sheet (4), but it serves as an additional check that the tubes have been correctly placed in the racks (2).

6. Test the donors' sera (Plate XIV). Place two rows of $2\times\frac{3}{8}$ inch tubes in the appropriate rack and transfer 1 vol. (0·08 ml.) of serum from each pilot tube to the corresponding $2\times\frac{3}{8}$ inch tube of the first row and 1 vol. into the corresponding tube of the second row. The work should proceed from left to right along the row, and as serum is removed from each pilot tube this tube should be placed in a hole in the pilot tube rack above that in which it was placed before; this prevents one from losing one's place. When this procedure is completed, all the tubes must be inspected to see that each has received its quota of serum. One vol. of a 3 per cent suspension of A cells is then placed in each tube of the first row, and of B cells in the second row. The A cells should be either A_1 cells or a mixture of the cells of not less than six group A individuals. (The use of A_2 cells tends to lead to an undue number of false negative results.) The tubes are then tapped to mix cells and serum and the racks put aside on the bench for one and a half hours at room temperature before reading.

7. Preparation of donor cell suspensions. From clotted blood samples in pilot tubes which have been properly "ringed" with a glass rod after clotting, and where the clot has retracted satisfactorily, it is possible to prepare separate serum and cell suspensions without the use of a centrifuge. Each pilot tube is taken in turn and the serum carefully poured off into another tube which is placed in position in the appropriate rack. The clot, and remaining serum, is then gently shaken from

which is liable to have led to errors in the determination of the groups of the other bottles.

15. **Inspection of sheets.** The person in charge of the grouping department inspects the sheets and assesses the accuracy of the day's work. The number of cases in which results with the donor's cells disagree with those with the donor's serum is considered according to what the donor's group is finally decided to be, and a careful watch is kept for any errors which might appear to be of a systematic character. For example, a number of false positive results with either the anti-A or anti-B serum might make one suspect that the reagent is infected, or mistakes which would be rectified if the results were reversed might make one suspect that the tubes had been interchanged with one another in the racks. It should always be borne in mind that a mistake which has come to light suggests the possibility of other mistakes which have not. Provided that there is no evidence of systematic mistakes and that the number of cases in which cells and sera disagree is small, the batch can be passed as satisfactory and the labelling of the bottles can proceed.

16. **Place the bottles of blood on the bench** so that the bottles of each group are together on a separate part of the bench. Three people are required for this. The first takes up each bottle in turn and calls out its number and the donor's name; the second person replies by calling out the appropriate group from the laboratory work sheet, whilst the third person listens to what is said and compares it with what is written on the blood collection sheet. This checks (14) since discrepancies between the two sheets would be noticed at this stage.

17. **Count the total number of bottles of each group,** according to the position of the bottles on the bench, and compare this with the total number of each group shown on the laboratory work sheet. This checks (16), but it is not a perfect check since it will not detect the situation in which two bottles are accidentally switched round with one another. Section (19) below constitutes a more effective check.

18. **Stick labels** showing the group on to the bottles according to their position on the bench.

19. **Replace the bottles** in the refrigerator. This is done in group order; one person takes up each bottle in turn and calls out its number, whilst the second and third persons check from the laboratory work sheet and from the blood collection sheet that the number corresponds with the group stated. All the bottles of one group are dealt with before proceeding to a second group. This is primarily a check of (16) and (18), but it is also a second check of (14).

The above procedure consists basically of five steps:

i. Putting the pilot tubes in order in the racks (2).
ii. Entering the serial numbers on to the laboratory work sheet (4).

cells, those which give a negative result with anti-A and a positive result with anti-B are set aside for a further period of one and a half hours, so that the cells can sediment again and they are then read microscopically. The serum of all apparently group B donors is also retested with A_2 cells. (The reason for this procedure is given on p. 108.) In the misdiagnosis of A_2B as B, the incorrect results with the donor's cells and with the donor's serum are connected with one another in a systematic manner and not merely associated with one another in a random manner, as is assumed to be the case in all other possible causes of error. This means that although both cells and serum have been tested they have not been tested independently of one another so that there has been no real check of the test. If a test and its checking process are not independent of one another the check is invalidated. This is one reason why a special test is necessary to avoid this particular error and why it cannot be allowed for in the statistical control of the accuracy of the grouping process described below.

12. **Recording the groups.** The two sheets are now brought together in a loose-leaf binder which ensures their correct alignment, and the group of each donor is recorded in a separate column. This should preferably not be done by the persons carrying out the tests. It may with advantage be done by the person in charge of the department. In all cases where the results with donor's cells are in disagreement with the results with donor's serum the test must be repeated, and it is best to read these tests microscopically. If there is still disagreement, further investigation will be needed to determine the group (see p. 118).

13. **Check** (12) by one person calling out each group according to the serological results, whilst a second person notes what group has been recorded in the appropriate column.

14. Copy the groups from the **laboratory worksheet** (Fig. 3) on to the **blood collection sheet** (Fig. 2). Discrepancies between the groups, as determined by the present tests and the stated group as shown by the previous records, will come to light at this stage. This is a most valuable check, and any discrepancies found must be investigated forthwith. None of the blood tested can be considered safe to use until this has been done, since, if one bottle of blood is incorrectly grouped, it should be presumed that others might have been wrongly grouped also until some explanation of the error is forthcoming. In such cases we check that the stated group as shown on the blood collection sheet agrees with the group as stated on the donor's record card, inspect the results of previous tests on the donor's blood in the laboratory records and test the group of the blood inside the bottle if necessary. In many cases the discrepancy proves to be due to a clerical error or confusion with another donor of the same name, but in any case the discrepancy must be reported to a senior technical person, who should inspect all the relevant documents and consider whether anything has occurred

to calculate the probable rate of error, as shown by the following example:

(a) Errors detected in placing pilot tubes in rack 9
(b) „ „ „ recording serial numbers on work sheet 1
(c) „ „ „ „ ABO group from serological results 8
(d) „ „ „ labelling the bottles of blood 16

Number of samples tested, 6,422

∴ probable rate of error of the whole process:

$$= \left(\frac{9}{6422}\right)^2 + \left(\frac{1}{6422}\right)^2 + \left(\frac{8}{6422}\right)^2 + \left(\frac{16}{6422}\right)^2$$

$$= \frac{9^2+1^2+8^2+16^2}{6422^2}$$

= 9·7 per million (or 1 in 103,000).

For the sake of simplicity, no account has been taken in this calculation of the additional checks incorporated in the system; it is calculated on the basis that each step is only checked once, and the additional checks form an additional safeguard.

Furthermore, it must be remembered that any calculation, relating to a process carried out by human beings where all sorts of difficulties can occur is of necessity an approximation and involves a number of assumptions. This does not mean, however, that the calculation is not useful even though it is only approximate.

(ii) CALCULATION OF THE ERROR INVOLVED IN THE SEROLOGICAL DETERMINATION OF THE ABO GROUP. This is calculated from the number of occasions on which results with the donor's cells do not agree with those with the donor's serum in the initial test, and the calculation only applies to the results with anti-A, anti-B, A cells and B cells. The use of group O serum constitutes an additional check of the serological determination of the ABO group, and is not included in the calculation. If the results with cells and serum disagree, it means that one of the original results was incorrect, and we can decide which it was when the donor's correct group has been finally determined. For example, if the initial test gave these results:

Anti-A	*Anti-B*	*A cells*	*B cells*
+	−	+	+

and the donor's group proved, on re-checking, to be group O, this would constitute a false positive result with the anti-A serum. Cases where the test is haemolysed do not count as a false result, since they do not lead to an incorrect reading but to the test having to be repeated. Cases where an anti-P or other antibody in the donor's serum is

iii. Serological determination of the group (6–11).
iv. Deducing the group from the serological results (12).
v. Sticking labels on the bottles (16 and 18).

The system provides for at least one independent check of each of these processes and there are additional checks of some of them. An undetected error in any of these five steps can lead to an incorrectly labelled bottle of blood. The above procedure has been built up over the years and almost every step in it has been introduced, at one time or another, as a result of our experience of the mistakes which occur in practice. We are convinced that some such equally detailed scheme is essential for success in large-scale ABO grouping.

Statistical Control

We shall now consider what steps can be taken to ascertain that a satisfactory standard of accuracy is being maintained in carrying out the procedure detailed above.

Each of the five basic steps, enumerated above, has its own rate of error, and since an error at any one of these five stages can, if undetected, lead to an incorrectly grouped bottle of blood, it follows that *the rate of error for each step can be summated to give the rate of error* for the whole process. The calculation of the rate of error may be divided into two parts: the calculation of the rate of error in the serological determination of the group, and the calculation of the rate of error involved in the four manipulative processes. It is best to consider the latter first.

(i) CALCULATION OF THE ERROR INVOLVED IN THE FOUR MANIPULATIVE PROCESSES. If a single step in the grouping procedure has a rate of error of, say, one in a thousand, and the corresponding checking process also has a rate of error of one in a thousand, then by chance an *error will remain undetected by the checking process once in a million* times. What we require to determine is the actual frequency with which *this is likely to occur. This cannot be determined by direct observation,* since the rate should be too low for this. Furthermore, we are not certain that a single error would ever come to light at all. The only way to arrive at any figure for the final error is by considering the frequency with which the checking process detects errors in the initial process. There is no means of telling the accuracy of the checking process, so that we have to arbitrarily assume that its accuracy is the same as that of the initial process which it is designed to check. If this is so, it means that the final rate of error can be obtained by squaring the frequency with which the checking process detects errors in the original manipulation. If a note is kept of the number of times that an error is revealed by the checking process, it is a simple matter

of all samples tested, and it is not easy to see how often this will coincide with a false positive result with the A cells.

Although

$$\frac{15}{6422}$$

of *all samples tested* gave false positive results with the A cells, only 44·05 per cent of these samples were A or AB, so that the frequency of false positive results with A cells *amongst group A and group AB bloods* is

$$\frac{15}{6422 \times 0{\cdot}4405}$$

Since all cases where a false negative result with the anti-A occurred must be of group A or group AB, the frequency with which the two errors will coincide is:

$$\frac{9 \times 15}{6422^2 \times 0{\cdot}4405}$$

of all samples tested.

Failure to detect the B antigen will similarly occur in:

$$\frac{2 \times 6}{6422^2 \times 0{\cdot}1155}$$

of all samples tested.

The frequency of incorrect diagnosis of the antigens when they are not present is calculated in the same way, taking into account the frequencies of bloods lacking the antigens. The frequency of incorrect diagnosis of the A antigen is thus:

$$\frac{(\text{false positives with anti-A}) \times (\text{false negatives with A cells})}{(\text{samples tested})^2 \times (\text{frequency of blood lacking the A antigen})}$$

or:

$$\frac{6 \times 10}{6422^2 \times 0{\cdot}5595}$$

and of incorrect diagnosis of the B antigen:

$$\frac{3 \times 10}{6422^2 \times 0{\cdot}8845}$$

The total error in the serological determination of the group is, of course, the sum of these four component errors, i.e.

$$\frac{1}{6422^2} \times \left(\frac{9 \times 15}{0{\cdot}4405} + \frac{2 \times 6}{0{\cdot}1155} + \frac{6 \times 10}{0{\cdot}5595} + \frac{3 \times 10}{0{\cdot}8845}\right)$$

$$= 13.5 \text{ per million (or 1 in 74,000)}$$

(iii) THE TOTAL RATE OF ERROR. This is, of course, the total of the two separate sources of error, manipulative and serological, calculated above. In the present example, it works out at:

23·2 per million samples tested (or 1 in 43,000).

responsible for the difficulty should, on the other hand, be counted as false positive results, even though the result obtained is obtained again on repeating the test. The reason for this is that we are not concerned at this stage with the cause of the discrepancy, but only with the frequency with which discrepancies occur. The actual calculation can best be understood by reference to the figures obtained in a sample period. These were as follows:

	False positive	*False negative*
Anti-A with donor's cells	6	9
Anti-B " " "	3	2
A cells with donor's serum	15	10
B " " " "	6	10

Total samples tested: 6,422.

There are four ways in which it is possible to get an ABO group wrong, i.e.

i. Failure to detect the A antigen
ii. " " " B "
iii. Falsely diagnosing an A antigen when none is present
iv. " " a B " " "

and it is necessary to calculate separately the chance of making each of these mistakes.

Failure to detect the A antigen occurs when a false negative result with the anti-A accidentally coincides with a false positive result with the A cells. However, the frequency with which this happens is not, as might be thought, simply the product of the frequencies of each separate error. The reason for this is that these two false results can only occur when the A antigen is, in fact, present; if it is absent they would be true results.

The group frequencies in north-west England are:

	%
O	47·45
A	41·0
B	8·5
AB	3·05

from which the following figures may be deduced:

	%
Bloods carrying the A antigen	44·05
" " B "	11·55
" lacking the A "	55·95
" " B "	88·45

Now, a false negative result with the anti-A occurred in

$$\frac{9}{6422}$$

of all samples tested, and it is not easy to see how often this will coincide with a false positive result with the A cells.

Although

$$\frac{15}{6422}$$

of *all samples tested* gave false positive results with the A cells, only 44·05 per cent of these samples were A or AB, so that the frequency of false positive results with A cells *amongst group A and group AB bloods* is

$$\frac{15}{6422 \times 0{\cdot}4405}$$

Since all cases where a false negative result with the anti-A occurred must be of group A or group AB, the frequency with which the two errors will coincide is:

$$\frac{9 \times 15}{6422^2 \times 0{\cdot}4405}$$

of all samples tested.

Failure to detect the B antigen will similarly occur in:

$$\frac{2 \times 6}{6422^2 \times 0{\cdot}1155}$$

of all samples tested.

The frequency of incorrect diagnosis of the antigens when they are not present is calculated in the same way, taking into account the frequencies of bloods lacking the antigens. The frequency of incorrect diagnosis of the A antigen is thus:

$$\frac{(\text{false positives with anti-A}) \times \qquad (\text{false negatives with A cells})}{(\text{samples tested})^2 \qquad \times (\text{frequency of blood lacking the A antigen})}$$

or:

$$\frac{6 \times 10}{6422^2 \times 0{\cdot}5595}$$

and of incorrect diagnosis of the B antigen:

$$\frac{3 \times 10}{6422^2 \times 0{\cdot}8845}$$

The total error in the serological determination of the group is, of course, the sum of these four component errors, i.e.

$$\frac{1}{6422^2} \times \left(\frac{9 \times 15}{0{\cdot}4405} + \frac{2 \times 6}{0{\cdot}1155} + \frac{6 \times 10}{0{\cdot}5595} + \frac{3 \times 10}{0{\cdot}8845}\right)$$

$= 13.5$ per million (or 1 in 74,000).

(iii) THE TOTAL RATE OF ERROR. This is, of course, the total of the two separate sources of error, manipulative and serological, calculated above. In the present example, it works out at:

23·2 per million samples tested (or 1 in 43,000).

Assessment of the Safety of the Process and Need for a Further Test

The calculation described above gives a valuable indication of the standard of accuracy of the work, and, if carried out at intervals, enables one to assess this and to take suitable steps when the rate of error rises.

It is important to realize that some rate of error is inevitable and each laboratory ought to consider what rate of error is acceptable to them according to their own particular circumstances.

It should also be borne in mind that these calculations are, of necessity, somewhat over-simplified, and do not take into account all the factors involved in the occurrence of errors. The calculations presuppose that the system is being correctly adhered to and that results and rates of error are correctly and honestly recorded. This is, unfortunately, not always the case. Also, for reasons given above, the mis-diagnosis of A_2B bloods with anti-A_1 in the serum as group B is an error which cannot be allowed for in the calculations. On the other hand there are two additional safeguards which will reduce the incidence of incorrectly grouped bottles of blood below that indicated by the calculations. The first, and in our experience the most valuable of these, is to compare the results with those obtained when the donors were previously bled, and the second is the use of group O serum which serves as an additional check in the serological determination of the ABO group but not, of course, as a safeguard against the manipulative errors.

Finally, in view of the fact that the seriousness of errors in the ABO grouping of donors can scarcely be overestimated, we have to consider whether the situation can be considered safe even though all the precautions described above have been carried out. Even if a rate of error of only 1 in 43,000 samples tested, as in the above example, could be persistently maintained, this might still represent more than one incorrectly grouped bottle of blood per annum. It is true that there are two additional safeguards; the records of previous results, and the use of group O serum, but it might be considered that these would only be sufficient to compensate for occasional departures from the routine laid down and for the way in which the errors are distributed. Errors may not always be randomly distributed, so that an accumulation of errors on one day may render the situation dangerous without necessarily showing in the final figures.

The cross matching test, of course, constitutes a further check of the donor's ABO group before the blood is given to the patient, but even so we do not feel that the standard of accuracy which it is practicable to achieve in large-scale ABO grouping is such that further tests can be dispensed with, and it is our opinion that a further test of the donor's group ought to be carried out in such a manner as to be independen

of the whole of the above process. This may be done by testing the donor's group when he attends the blood collection session or by having a second pilot tube attached to each bottle, which is tested at some time before the blood is used. A test of the donor's cells with anti-A and anti-B on microscope slides is likely to be of sufficient accuracy to serve the purpose of this further check, but, however the test is done, we do not feel that it ought to be omitted.

CHAPTER VIII

THE Rh GROUPS

The Specific Reactions of the Rh Antigens and Antibodies

THE Rh blood groups comprise the antigens C, D, E, c, e, f, V and their corresponding antibodies, together with the hypothetical antigens d, F and v. In addition, other forms of the antigens C, D and E are known with specific antibodies in some cases. It is necessary to consider the antigens C, D, E, c, d, e first.

THE ANTIGENS C, D, E, c, d, e

Wiener considers that there are eight alleles at a single locus, so that the antigens C, D, E, c, d, e (which he calls rh', Rh_0, rh", hr', Hr_0 and hr" respectively) are determined in threes by a single gene. Fisher and Race, on the other hand, consider that the occurrence of these antigens on the red cell surface is determined by three pairs of closely linked genes, each having two alternative alleles, C-c D-d and E-e. There are thus eight possible types of chromosome. Wiener's eight genes correspond, of course, to the eight chromosomes of Fisher and Race, so that from a practical point of view it does not make a great deal of difference which theory one adheres to. It is, however, unfortunate that three different notations are in use for the Rh groups. These are the CDE notation, the Rh-Hr notation, and the so-called "clinical notation" much used in Great Britain for verbal and other day-to-day purposes.

TABLE 41. THE EIGHT Rh CHROMOSOMES

CDE notation	*Clinical notation*	*Rh-Hr notation*	*Approximate frequency in white population*
CDe	R_1	R^1	Common
cDE	R_2	R^2	Common
CDE	R_z	R^z	Rare
cDe	R_0	R^0	2%
Cde	R'	r'	1%
cdE	R"	r"	1%
CdE	R_y	r^y	Very rare
cde	r	r	Common

Table 41 shows the eight chromosomes together with a much simplified and easily memorized list of their frequencies. This table also enables one to work out which antibodies react with the erythrocytes of persons having any of these chromosomes; e.g. anti-C reacts when

the chromosomes R_1, R_2, R′ or R_y are present. Since each individual has a pair of Rh chromosomes there are thirty-six possible Rh genotypes, and the simplified list of frequencies in this table enables one to form an estimate of their approximate frequencies. For example, R_1R_2 and rr are each composed of two common chromosomes and must, therefore, be common genotypes. R_1R' and R″r are each composed of a common and of a less common chromosome and so must have a lower frequency. Similarly, it can be seen that R′R″ and R_zr will not be encountered often and, whilst R_yr is very rare, R_yR_y must be very, very rare indeed. An understanding of this is important when we come to determine a person's Rh group. Suppose, for example, that a blood sample gave the following reactions:

Anti-C	.. .. ..	−
„ D	.. .. ..	+
„ E	.. .. ..	+
„ c	.. .. ..	+
„ e	.. .. ..	+

This means that the antigen C is absent and the antigens D, E, c and e are present. We have no knowledge of d since the hypothetical anti-d is not known. Reference to Table 112 will show that the genotype could be cDE/cde, cDE/cDe or cDe/cdE, which we more conveniently refer to as R_2r, R_2R_0 and R_0R'' respectively. There is no way of telling by serological, as opposed to genetical, means which of these three alternatives is correct but a consideration of the frequencies shows that some are more probable than others. The genotype R_2r, being composed of two common chromosomes (Table 41), must be of common occurrence, R_2R_0 by a similar argument is less common, whilst R_0R'' is rare. In ordinary parlance, such a sample would be referred to as "R_2r" and by the term "R_2r" we mean a blood giving the reactions shown above and coming from a person whose genotype is probably R_2r but might be R_2R_0, or, although this is less likely, R_0R''.

Of course, in some cases uncertainty does not arise, as, for example, rr, where blood of no other genotype will give the appropriate reactions. Occasionally, bloods which have only been tested with anti-C, anti-D and anti-E have to be referred to, and in this case we would refer to [illegible] by R′ blood [illegible] with anti-C [illegible] tested with anti-c. It might be of genotype R′r or R′R′. This system refers to blood by its "probable genotype" and confusion is unlikely provided it is remembered that they are only the most likely genotypes and not necessarily the actual genotypes and that the term used only represents the results of the tests. The rule is to refer always to the most probable of the possible alternative genotypes whilst constantly bearing the alternatives in mind. A list of the actual groups of blood encountered,

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Wiener considers that there are eight alleles at a single locus, so that the antigens C, D, E, c, d, e (which he calls rh′, Rh_0, rh″, hr′, Hr_0 and hr″ respectively) are determined in threes by a single gene. Fisher and Race, on the other hand, consider that the occurrence of these antigens on the red cell surface is determined by three pairs of closely linked genes, each having two alternative alleles, C-c D-d and E-e. There are thus eight possible types of chromosome. Wiener's eight genes correspond, of course, to the eight chromosomes of Fisher and Race, so that from a practical point of view it does not make a great deal of difference which theory one adheres to. It is, however, unfortunate that three different notations are in use for the Rh groups. These are the CDE notation, the Rh-Hr notation, and the so-called "clinical notation" much used in Great Britain for verbal and other day-to-day purposes.

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CDE	R_z	R^z	Rare
cDe	R_0	R^0	2%
Cde	R′	r′	1%
cdE	R″	r″	1%
CdE	R_y	r^y	Very rare
cde	r	r	Common

Table 41 shows the eight chromosomes together with a much simplified and easily memorized list of their frequencies. This table also enables one to work out which antibodies react with the erythrocytes of persons having any of these chromosomes; e.g. anti-C reacts when

who are immunized by R_1 may produce either anti-D alone or anti-C+D. It is very exceptional for them to produce anti-C without anti-D (see p. 176).

The commonest Rh antibodies encountered in the sera of D positive persons are anti-E and anti-c, and anti-E is found about twice as often as anti-c.

Anti-c is formed by R_1R_1 individuals. Sometimes it is produced on its own and sometimes it is associated with anti-E. The presence of anti-E in anti-c sera is not easy to detect, since only rare types of blood, such as R_1R_z and R_1R_y, contain the antigen E without also containing the antigen c. All anti-c sera should, therefore, be suspected of containing anti-E unless it is known that the person forming the antibody has never been exposed to the E antigen or unless the serum has been specially tested with R_1R_z or R_1R_y cells. It may be noted that unless the true genotypes are known from family studies these cells themselves can only be detected by the use of an anti-c serum free from anti-E, since they are detected by the fact that they give positive results with anti-C, anti-D and anti-E sera but negative results with anti-c sera. There is thus the danger of getting into a vicious circle unless one has a known pure anti-c serum to start with. However, this is not really a point of any great practical importance since the main purpose for which anti-c is required is to determine whether R_1 bloods are R_1R_1 or R_1r and for this purpose the presence of anti-E is irrelevant. Anti-c sera also sometimes contain anti-f, but there is no known test cell carrying the antigen f, and not carrying the antigen c, which one could use to show this. It can accordingly only be demonstrated by first removing the anti-c by absorption with c positive, f negative cells such as R_2R_2 and then testing with f positive cells such as rr. The possible presence of anti-f does not interfere with the use of anti-c sera for distinguishing R_1R_1 from R_1r, but it must always be considered in more advanced investigations.

Anti-E may be formed in the serum of R_1R_1 persons, in which case it is commonly associated with anti-c, or in the serum of R_1r persons, in which case one can be sure that anti-c is absent. Whilst it is difficult to detect anti-E in an anti-c serum, it is very easy to detect anti-c in an anti-E serum, since one has only to test with rr cells to show its presence.

Anti-e and anti-C are formed much less commonly, anti-e being encountered mainly as an autoantibody in the serum of cases of acquired haemolytic anaemia. Many anti-e sera contain anti-C and anti-f also, and the detection of these presents just the same problems as the detection of anti-E and anti-f in anti-c sera. Indeed, the sera anti-c+E and *anti-e+C* seem in some ways to have an antithetical relationship.

Apart from anti-d, which remains a hypothetical entity, anti-C is the rarest of the Rh antibodies to be found alone, and this antibody,

together with their frequencies and possible alternative genotypes, is given in Table 112 and the frequency of each group is the frequency of bloods giving each serological pattern. The actual serological results and the genotype frequencies are also shown.

This system of Rh nomenclature is widely used in the day-to-day work of a large number of laboratories and is found to be unambiguous in practice. We have, therefore, thought it best to keep to this system in this book. There are, however, a number of alternative systems in use. *The best known of these are the Rh-Hr system and the CDE system.* The first of these, Wiener's system, has a separate set of symbols for phenotypes (Rhrh, Rh_1Rh_2, etc.) and for genotypes (R^1r, R^1R^2, etc.) which is advantageous in some ways, but it is difficult to remember which phenotypes comprise which genotypes and which phenotypes react with which sera. In Wiener's system the antibodies are named as follows: anti-C = anti-rh′, anti-D = anti-Rh_0, anti-E = anti-rh″, anti-c = anti-hr′, anti-d = anti-Hr_0, anti-e = anti-hr″. The second system consists of keeping entirely to the letters C, D, E, c, d, e, and referring to the genotypes and most likely genotypes, as, for example, CDe/cde. This has certain advantages but is very cumbersome when written and hopelessly so when spoken.

THE ANTIBODIES, ANTI-C, D, E, c, e

The antibodies may be considered from two points of view, from the point of view of their specificity and from the point of view of their mode of reaction, i.e. whether agglutinins or incomplete antibodies. We shall consider the specificity first; the mode of reaction is considered in a later section.

Whilst in theory antibodies can be formed against any of the antigens which a person forming the antibody does not himself possess, in practice it is found that certain antibodies and certain combinations of antibodies are encountered much more frequently than others. This is due to some extent to the different frequencies of the various Rh groups. Anti-D is much the most important and commonest antibody encountered, and in many cases the only antibody which needs to be considered. D negative persons will form anti-D if transfused with D positive blood so frequently that it is necessary to take steps to avoid doing this by D typing all prospective recipients. Similarly, this is the antibody responsible for the great majority of cases of haemolytic disease of the newborn. This is the reason for D typing antenatal patients so that a search for antibodies can be made in the serum of those who are D negative. D negative persons most commonly form anti-D alone, but anti-C+D is also common. Anti-D+E, on the other hand, is found only rarely, and anti-C+D+E is very rare. It will be noticed that people do not necessarily produce all the antibodies of which they are theoretically capable; so that, for example, rr persons

TABLE 42. ANTI-C SERA WITH CELLS WITH VARIOUS C ANTIGENS

Type of serum	*Agglutinating anti-C+incomplete anti-D*									*Anti-C^w*	*Anti-C^x*
Method of test	*Saline agglutination 37° C.*									*Albumin 37° C.*	*Albumin 37° C.*
Reciprocal of dilution	1	2	4	8	16	32	64	128	256	1	1
Cells:											
R_1r	v	v	v	++	+	w	—	—	—	—	—
R_1r	v	v	v	+++	++	w	—	—	—	—	—
Cells:											
R_1r .. .. (C^xDe/cde)	++	+	w	—	—	—	—	—	—	—	v
R_1r .. .. (C^wDe/cde)	+	w	—	—	—	—	—	—	—	v	—
R_1r .. .. (C^uDe/cde)	++	+	—	—	—	—	—	—	—	—	—

in some ways to the Strong D cells described below. We have not, unfortunately, had the opportunity of investigating this matter exhaustively, but have observed that certain R′r bloods react more strongly than others. On one occasion, a saline agglutinating anti-D serum, which had been tested with R′ and which was, therefore, thought to be free from anti-C agglutininin, was found to give a positive result with an R′r blood. At first we wondered whether this R′r was really R_1^ur, but this proved not to be so, and further investigation showed that the anti-D serum in question would agglutinate R′ papainized cells. It would not, however, agglutinate normal R′ cells in saline except in the case of the "strong" R′ with which we were dealing. The donor of the anti-D serum subsequently developed a normal anti-C. We have encountered the same phenomenon on two or three occasions subsequently and the lesson to be learned from it is that if an anti-D serum gives a negative result with R′ cells it does not necessarily mean that it will give a negative result with all samples of R′ cells.

THE ANTIGENS, D^u, STRONG D, AND —D—

Just as the C antigen can exist in a number of different forms so can the D antigen also. These comprise the various types of D^u blood which react more weakly than the normal D antigen, the various Strong D bloods which react more strongly than the normal D antigen [illegible] D. [illegible] C, c, E, e. The D antigen ordinarily encountered, which may be conveniently referred to as "normal D", thus lies in the middle

whilst of common occurrence in D negative persons in association with anti-D, is to be found only very rarely on its own in the serum of D positive persons. It is a curious and unexplained fact that, whilst anti-C+D is a good deal more common than anti-D+E in the serum of D negative persons, anti-C is a great deal rarer than anti-E in the serum of D positive persons. In the case of D negative persons this is to some extent due to the frequency of the Rh types, since such people are more likely to receive an antigenic stimulus from R_1 than from R_2 blood. But in the case of Rh positive persons no such argument can be considered, since a little reflection will show that the number of R_1 individuals stimulated by R_2 blood is precisely the same as the number of R_2 individuals stimulated by R_1 blood.

All these antibodies may be found as saline agglutinins or incomplete antibodies or as combinations of these, so that such mixtures as *agglutinating anti-C + agglutinating anti-D, agglutinating anti-*C+incomplete anti-D, and agglutinating anti-E+incomplete anti-c are seen. The properties of agglutinating and incomplete antibodies and the means of detecting them are discussed elsewhere.

THE ANTIGENS C^w, C^x, C^u, c^v

In certain bloods the C antigen exists in various forms reacting differently from the C antigen ordinarily encountered. This is shown in two ways. Firstly it is shown by the fact that if the cells concerned are tested with a number of anti-C sera they are only agglutinated by some of them, and, furthermore, if these sera are titrated against the modified C cells a much lower titre than with normal C cells is found in many cases. Secondly the difference is shown by the existence of the specific antibodies, anti-C^w (Callender and Race, 1946) and anti-C^x (Stratton and Renton, 1954), which only react with cells carrying the C^w and C^x antigens respectively. These antibodies can be, and indeed usually are, formed by C positive individuals. An apparently naturally occurring anti-C^x has been found in the serum of a woman whose husband was C^x negative, and who had never received a transfusion of C^x positive blood (Plaut *et al.*, 1958). Specific antibodies only reacting with C^u and c^v cells are unknown. C^u can only be demonstrated by means of its weak and irregular action with anti-C sera. The antigen c^v reacts similarly with anti-C sera, but it is thought to have also some affinity with c, which is the reason for the choice of symbol c^v (Race, Sanger and Lawler, 1948a; Race and Sanger, 1951).

Table 42 shows titration results with an anti-C serum with a number of cells with modified C antigens, and also the reactions of these antigens with their specific antisera.

From time to time we have encountered what may well be a further modification of the C antigen reacting more strongly with anti-C sera than the C antigens ordinarily encountered, and perhaps analogous

TABLE 42. ANTI-C SERA WITH CELLS WITH VARIOUS C ANTIGENS

Type of serum	*Agglutinating anti-C+incomplete anti-D*									*Anti-C^w*	*Anti-C^x*
Method of test	*Saline agglutination 37° C.*									*Albumin 37° C.*	*Albumin 37° C.*
Reciprocal of dilution	1	2	4	8	16	32	64	128	256	1	1
Cells:											
R_1r	v	v	v	++	+	w	—	—	—	—	—
R_1r	v	v	v	+++	++	w	—	—	—	—	—
Cells:											
R_1r .. .. (C^xDe/cde)	++	+	w	—	—	—	—	—	—	—	v
R_1r .. .. (C^wDe/cde)	+	w	—	—	—	—	—	—	—	v	—
R_1r .. .. (C^uDe/cde)	++	+	—	—	—	—	—	—	—	—	—

in some ways to the Strong D cells described below. We have not, unfortunately, had the opportunity of investigating this matter exhaustively, but have observed that certain R′r bloods react more strongly than others. On one occasion, a saline agglutinating anti-D serum, which had been tested with R′ and which was, therefore, thought to be free from anti-C agglutininin, was found to give a positive result with an R′r blood. At first we wondered whether this R′r was really $R_1^u r$, but this proved not to be so, and further investigation showed that the anti-D serum in question would agglutinate R′ papainized cells. It would not, however, agglutinate normal R′ cells in saline except in the case of the "strong" R′ with which we were dealing. The donor of the anti-D serum subsequently developed a normal anti-C. We have encountered the same phenomenon on two or three occasions subsequently and the lesson to be learned from it is that if an anti-D serum gives a negative result with R′ cells it does not necessarily mean that it will give a negative result with all samples of R′ cells.

THE ANTIGENS, D^u, STRONG D, AND —D—

Just as the C antigen can exist in a number of different forms so can the D antigen also. These comprise the various types of D^u blood which react more weakly than the normal D antigen, the various Strong D bloods which react more strongly than the normal D antigen, and homozygous —D—/—D— bloods which exhibit the most strongly reacting D antigen of all, and which in addition show no evidence of the antigens C, c, E or e. The D antigen ordinarily encountered, which may be conveniently referred to as "normal D", thus lies in the middle

of a range of D antigens of different strengths, extending from low grade D^u on the one hand to —D—/—D— on the other, and this range appears to be continuous at any rate as far as Strong D. There is some variation in the strength of D antigen even amongst the normal D cells themselves, and this is partly, but not entirely, dependent on the Rh genotype. R_2R_2 cells in particular usually react more strongly than cells of any other Rh genotype.

The effect of these variations in the strength of the D antigen shows itself in two ways. Firstly it affects the titre of an anti-D serum with the cells concerned, and secondly it affects the ability of the cells to be agglutinated in saline suspension by mixtures of agglutinin and incomplete antibody, i.e. by different anti-D sera. As discussed on p. 169, increasing antigen strength is only reflected by an increasing titre with the corresponding antiserum up to a certain point; above this point increasing antigen strength has very little effect on the titre. In the case of the various D antigens, this point corresponds to normal D so that D^u cells give lower titres with anti-D sera than do normal D cells, but Strong D and —D—/—D— cells show little or no increase of titre. Most anti-D sera consist of a mixture of saline agglutinin and incomplete antibody and these combine with the antigen on the red cell surface in a proportion which is dependent on the proportion in which these two types of antibody are present in the serum. This means that there is a competition for the available antigen on the red cell surface between the two types of antibody and this may result in the antigen being unable to take up a sufficient amount of agglutinin from any individual serum for agglutination in saline suspension to occur. The weaker the antigen on the cell surface the greater the chance that this will happen, and consequently the smaller the proportion of anti-D sera which will be able to agglutinate the cells in question. This means that as one goes up the range from low-grade D^u to —D—/—D— the proportion of anti-D sera able to agglutinate the cells in saline progressively increases. Anti-D sera are divided into saline agglutinating sera and incomplete sera on an arbitrary basis, i.e. by their ability to agglutinate normal D cells in saline, so that some agglutinating sera are unable to agglutinate D^u cells in saline, while some incomplete sera are able to agglutinate Strong D and —D—/—D— cells in saline. These considerations explain many of the reactions of the modified D antigens; which are summarized in Table 43. The modified D antigens differ from the modified C and E antigens in that no specific antibodies analogous to anti-C^w, anti-C^x and anti-E^w are known. The difference between one type of D antigen and another is largely of a quantitative nature, and the differences which are found between one modified D antigen and another are to a large extent explicable in terms of differences in their strengths of reaction with ordinary anti-D sera (but see p. 164).

TABLE 43. GENERAL REACTIONS OF THE D ANTIGENS

Test		Type of antigen				
Type of anti-D serum	*Medium*	*-D-/-D-*	*Heterozygous -D-* *Strong* D,R_1R_2	*Normal D*	*High grade* D^u	*Low grade* D^u
Incomplete	Saline	Almost all positive	A few positive	Negative	Negative	Negative
Saline agglutinating	Saline	All positive	All positive	All positive	Some positive	Negative
Incomplete (albumin agglutinating)	Albumin	All positive	All positive	All positive	Only a few positive	Negative
Saline agglutinating	Albumin	All positive	All positive	All positive	Many positive	Negative
Incomplete*	Coombs test	All positive	All positive	All positive	Almost all positive	Many positive

* Excluding those only active against papainized cells.

REACTIONS OF D^u (Stratton, 1946; Race *et al.*, 1948b, 1948c; Stratton and Renton, 1948, 1949; Renton, 1949; Renton and Stratton, 1950). The more strongly reacting D^u cells, known as high-grade D^u, are agglutinable in saline suspensions by only a proportion of saline agglutinating anti-D sera; their reactions are analogous to those of the modified C cells. The more weakly reacting D^u cells, known as low-grade D^u, are inagglutinable in saline except by those occasional sera which are both of high titre and which consist entirely, or almost entirely, of agglutinin (see p. 177). These low-grade D^u cells are detected by their reaction with incomplete anti-D sera in the Coombs test. In the case of the weakest members of the series, only the strongest incomplete anti-D sera are capable of giving a demonstrable reaction with the cells since their D-antigen is so weak that a high concentration of antibody is needed before the cells will take up sufficient to give a positive Coombs test. D^u cells are agglutinable in albumin suspension by some of the saline agglutinating anti-D sera which fail to agglutinate them in saline suspension, but they are not usually agglutinable in albumin by incomplete anti-D sera even if these are able to agglutinate normal D cells in albumin. In serum albumin the picture is similar, but a higher proportion of the sera give positive results.

D^u cells may prove antigenic to D negative persons, and haemolytic disease of the newborn due to anti-D has been known where the child was D^u, so that D^u should be considered as Rh positive for all practical purposes. On the other hand, two or three cases have been described where anti-D appeared in the serum of D^u individuals, though this would appear to be very exceptional.

TABLE 44. AGGLUTINATION OF STRONG D CELLS IN SALINE SUSPENSION BY SELECTED INCOMPLETE ANTI-D SERA

(After Renton and Hancock, 1956)

Cells	*Incomplete anti-D sera*						
	214	254	*Li*	291	*St*	*Ni*	*Ha*
Normal D:							
R_1R_1	−	−	−	−	−	−	−
R_1R_2	−	+	−	+	−	−	−
R_1r .. .	−	−	−	−	−	−	−
R_2r	−	−	−	−	−	−	−
Strong D:							
R_1r	v	+++	+++	v	v	+++	++
R_2R_2	++	−	−	−	+	−	−
R_1r	+++	++	++	++	+	+++	−
R_2r	+++	+	+	++	+	+	−

REACTIONS OF STRONG D (Renton and Hancock, 1955, 1956). Strong D cells absorb more anti-D than normal D cells, are agglutinated by *all saline agglutinating anti-D sera*, and are agglutinated by a few incomplete anti-D sera also (Table 44). Many R_2R_2 bloods behave similarly. The difference between these cells and normal D cells is most readily demonstrated by means of the blocking titration which is carried out by means of the following technique:

Technique of the Blocking Titration:

To each tube of a series of doubling dilutions of a suitable incomplete anti-D serum in saline add 1 volume of a 3 per cent saline suspension of the cells to be tested. The contents of the tube should be mixed and incubated at 37° C. for half an hour. One volume of a suitably diluted anti-D agglutinating serum is then added to each *tube, mixed, and the tube* incubated for a further one and a half hours at 37° C. The result is then read microscopically.

TABLE 45. THE BLOCKING TITRATION
(After Renton and Hancock, 1956)

Cells	*Reciprocals of dilutions of incomplete anti-D sera*						
	1	2	4	8	16	32	64
Normal D:							
R_2r	—	—	—	—	+	++	++
R_1R_1	—	—	—	—	—	—	—
R_1R_2	—	—	w	w	+	++	++
R_1R_1	—	—	—	—	—	w	+
Strong D:							
R_1R_2	w	++	++	++	++	+++	+++

Each tube of the blocking titration contains 1 volume of a dilution of incomplete anti-D and 1 volume of cell suspension, to which is subsequently added 1 volume of a saline agglutinating anti-D.

Table 45 shows some results obtained by this technique, and the rationale behind the test is that when more antigen is present on the cell surface a greater amount of incomplete antibody is needed to block the antigen against the agglutinin.

Strong D cells with modified C antigens have been known and sometimes there is evidence suggesting modification of the other Rh antigens also.

REACTIONS OF —D— (Race *et al.*, 1950, 1951; Waller *et al.*, 1953). —D—/—D— cells, as mentioned above, have the strongest D antigen of

all, and they are consequently agglutinated in saline suspension by almost all incomplete anti-D sera. The anti-D sera which fail to agglutinate them are presumably those containing an exceptionally high proportion of incomplete antibody to agglutinin, or sera containing very little agglutinin. These cells entirely lack the C, c, E, and e antigens, and they are of very infrequent occurrence. If —D—/—D— persons become immunized to Rh they are able to form anti-C, anti-c, anti-E and anti-e or combinations of these. Some of these combinations of antibodies will react with cells of all Rh types other than —D—/—D—. Heterozygous —D— cells have also been described. Their D antigen is not as strong as that of homozygous —D—/—D— cells, so that they are only agglutinated in saline suspension by a few incomplete anti-D sera. They closely resemble Strong D cells in their reactions, indeed it would appear that the distinction between them could only be made by family studies, by dosage studies, or by the presence of the C and c or E and e antigens.

Mosaic Structure of the D Antigen

Although most of the reactions of D^u, Strong D and —D— can be explained on a purely quantitative basis, qualitative differences between the D antigens of different individuals also exist. Detailed studies of D^u cells with a large number of anti-D sera (Renton, 1949) suggested this, but it is most clearly shown by the very occasional occurrence of anti-D in the serum of D positive individuals.

The serum of these people contains an anti-D which reacts with almost all D positive cells but not with the patient's own cells. Their cells react with all anti-D sera. This shows that the D antigen in these people is different from the D antigen of others. Individuals of type D^u with anti-D in the serum have also been described. Table 46 is abstracted from a table by Wiener *et al.* (1957) describing the reactions of a D positive person with anti-D in the serum. This case

Table 46. Mosaic Structure of the D Antigen
(Modified from a Table by Wiener *et al.*, 1957)

Serum	*Cells*		
	R_1R_1	*Ar*	*W*
Ar	64	0	4
W	512	8	0

Ar = Patient of Argall (D^u with anti-D in serum).
W = Patient of Wiener *et al.* (D with anti-D in serum).
Figures indicate titre with ficinated cells.

was cross-tested with a case of D^u which had an anti-D in the serum also, and it shows very clearly that the D antigens of these two individuals differ qualitatively from one another.

A similar case was described by Rosenfield *et al.* (1957) who very kindly sent us some blood from their patient. Some of our tests on this sample of blood are described below, since they serve to illustrate some of the features of such cases and some of the principles involved in their investigation.

The cells were of type R_0r and nothing unusual was noticed in their reactions with anti-C, E c or e sera. Moreover, the cells react, so far as is known, with all anti-D sera except that of the patient herself, so that the difference between the D antigen of these cells and a normal D antigen can only be shown by differences in the strength and manner of its reaction with anti-D sera. The cells of the patient (Co) were compared with those of a normal R_0r individual (Br). When these cells were tested in saline at 37° C. with thirty-eight agglutinating and *incomplete* sera, the following results were seen (the sera have, of course, been rearranged so as to show the effect more clearly).

Serum No.:	1	2	3	4	5	6	7	8	9	10	11	12
Cells Br ..	v	v	v	v	v	v	v	+++	+++	+++	+++	+++
Cells Co ..	v	v	v	v	v	v	+++	+++	+++	+++	+++	+++

Serum No.:	13	14	15	16	17	18	19	20	21	22	23	24	25
Cells Br	+++	+++	+++	++	++	+	+	+	+	–	–	–	–
Cells Co	w	w	–	+	w	+	–	–	–	–	–	–	–

Serum No.:	26	27	28	29	30	31	32	33	34	35	36	37	38
Cells Br ..	–	–	–	–	–	–	+	–	–	–	+	++	+++
Cells Co ..	–	–	–	–	–	–	++	+++	v	v	v	v	v

From the results with the first twenty-one sera one would think that Co was a high grade D^u, but the results with sera Nos. 22 to 38 are suggestive of Strong D or heterozygous —D— cells; indeed, sera 33, 34 and 35 might fairly be described as incomplete sera which agglutinate Co in saline suspension. In other words, with some sera Co behaves as if it had a weak form of the D antigen, and with others as if it had a D antigen stronger than normal. Absorption of serum No. 36 with normal D cells removed all reaction with Co confirming that the reactions are due to anti-D and not to some other antibody in the sera.

Cross-absorption and elution experiments revealed the nature of the difference between Co and Br more clearly.

An anti-D serum was absorbed with an equal volume of the cells Br and Co and eluates were also made, using the same serum and cells. Sera and eluates were titrated against papainized cells. The titres were:

Cells used in titration	*Sera*			*Eluates*	
	Unabsorbed	*Absorbed with Br*	*Absorbed with Co*	*Eluted from Br*	*Eluted from Co*
Br	128	32	64	64	32
Co	128	32	8	64	128

This is an example of a cross-reacting system. The serum has initially the same titre with Br as with Co and absorption with or elution from Br does not affect this equality. The cells Co, on the other hand, take up preferentially that part of the serum which reacts more strongly with them than with Br and this is released again on elution. This type of behaviour is a common feature of serological systems in which two similar cross-reacting antigens are present. It can be seen that absorption with either type of cell reduces the titre against the other and that an eluate from either type of cell reacts with the other, which shows that the two antigens cross-react. The fact that absorption with Co makes the serum have a higher titre for Br than for Co, whilst elution from Co has the reverse effect shows that there is a qualitative difference between the two antigens.

Now this particular serum has initially the same titre with Br as with Co, but this does not necessarily mean that it reacts equally well with both. Only in the case of weakly reacting antigens are differences in the strength of reaction shown by differences of titre (see p. 170). This serum reacts better with Co than with Br as is shown by the fact that absorption with Co reduces the titre against Co to a greater extent than absorption with Br reduces the titre against Br. Also the eluate from Co has a higher titre against Co than the eluate from Br against Br.

As discussed on p. 170, the agglutination of D positive cells in saline suspension by anti-D sera is dependent on the proportion of incomplete antibody to agglutinin which they contain. The more strongly reacting the antigen, the more readily will the cells be agglutinated by sera containing a high proportion of incomplete antibody to agglutinin. This is because the strongly reacting antigen is less easily blocked.

This serum reacts more strongly with Co than with Br, which means that the antigen of Co is more strongly reacting than that of Br so far as this serum is concerned, and thus it might happen that the proportion of incomplete antibody in the serum was high enough to prevent agglutination of Br but not of Co when the cells were tested in saline suspension (the titres given above were against papainized cells). This [illegible] good agglutination [illegible]

[illegible] some sera react better with Co and some better with Br, and the fact that some se

will only agglutinate Co in saline suspension and others will only agglutinate Br is simply a reflection of their strength of reaction with the two types of cells.

THE ANTIGENS, E^u, E^w (Ceppellini *et al.*, 1950; Ceppellini, 1950; Greenwalt and Sanger, 1955)

These are precisely analogous to the antigens C^u and C^w; they give weak reactions and low titres with anti-E sera and a specific antibody, anti-E^w, is known reacting only with E^w cells. Anti-E^u is not known.

THE OTHER Rh ANTIGENS, f, V (Rosenfield *et al.*, 1953; Sanger *et al.*, 1953; De Natale *et al.*, 1955; Giblet *et al.*, 1957)

A pure anti-f has been found in the serum of an R_1R_2 individual. It reacted with the cells of all persons having the chromosomes r or R_0, but with no others. Anti-f is often present in anti-c sera and sometimes in anti-e sera also (p. 157).

The first example of anti-V was found in the serum of a white male, rr, patient who had been transfused with Negro blood. It reacted with almost a third of the Negro bloods tested, but only with 0·5 per cent of white bloods. All the positive bloods came from individuals with the chromosomes r or R_0. The precise place of V in the Rh system is not yet clear.

The Mode of Reaction of Rh Antibodies

THERMAL OPTIMUM

The great majority of Rh antibodies act best at 37° C. and only weakly, if at all, at 16° C., but very occasionally Rh antibodies are encountered which act better at the lower temperature. Some of these cold Rh antibodies are of immune origin, such as the cold anti-C antibody referred to below, and others seem to be of natural occurrence, such as certain anti-E sera which have been found acting best at low temperature and where there is no history of antigenic stimulation.

TYPES OF Rh ANTIBODY

The Rh antibodies in different sera react with erythrocytes in a variety of different ways. Some agglutinate the cells in saline suspension, others only in albumin suspension or by the Coombs test, and some are only detectable by the use of papain treated cells (see Table 55, p. 188). If the albumin and Coombs tests are compared as a means of detecting antibodies it is found that whilst many react by both methods, a number react only in the Coombs test, and a few only in the albumin test. Differences such as these lead one to suppose that a variety of types of antibody of the same specificity exists, but the fact that it is not known precisely how many types of antibody there

are, and which types react in which tests, renders the whole subject rather confusing.

The first distinction which was drawn was between the saline agglutinin which agglutinates cells in saline suspension, and the incomplete antibody which fails to do so and is only detectable by other means. Even this first distinction, however, is not always as clear cut as at first sight seems to be the case, since some of the tests for incomplete antibody give positive results with agglutinin also, and since incomplete antibody has a blocking effect which may interfere with the agglutinating effect of the agglutinin, so that the fact that a serum fails to agglutinate cells in saline suspension does not mean that the serum is necessarily free from agglutinin.

Incomplete antibodies have themselves been thought to be non-homogeneous and, as a result of discrepancies between results given by different tests for incomplete antibody and by various physical and chemical fractionating procedures, incomplete antibodies have been divided into a number of types which have been referred to by terms such as "agglutinoid", "cryptagglutinoid", "blocking antibody" and "albumin-agglutinin". However, the complications which occur even in the case of the simple division into saline agglutinin and incomplete antibody have even more force when one attempts to subdivide incomplete antibody into different types. Another difficulty is that the tests vary in sensitivity so that some of the differences are of a purely quantitative nature. As a result of these difficulties, no clear picture of the different types of incomplete antibody which exist has emerged, though there is no doubt of the existence of more than one type.

The existence of various ill-defined types of incomplete antibody has, however, little practical importance, and, provided it is remembered that the different tests for incomplete antibodies do not always detect exactly the same thing, it can generally be ignored. The widespread use of saline agglutination techniques, on the other hand, means that the distinction between saline agglutinin and incomplete antibody is of considerable importance. These two types of antibody compete with one another for the antigen on the cell surface, and under some circumstances the presence of incomplete antibody can accordingly interfere with the action of the agglutinin. The extent of this interference is in turn influenced by the properties of the antigen involved and the interaction of these factors gives rise to effects such as zoning and blocking. It also determines which types of cell a serum is able to agglutinate and may affect its ability to show dosage.

Some of the ways in which these factors operate are considered below. They have been most intensively studied in relation to the reaction of anti-D with the different types of D antigen, but agglutinins and incomplete antibodies of other specificities are well known, and there is no reason to suppose that they behave differently from anti-D.

Effect of Antigen on Agglutinin Titre

The titre of an anti-D agglutinating serum with D positive cells is dependent not only on the amount of agglutinin in the serum but also on the type of D antigen borne by the cells. The titre with —D—/—D—, Strong D and R_2R_2 cells is almost the same as that with normal D cells, whilst the titre with D^u cells is less than that with normal D cells. This effect is shown in Table 47 and the reason for it appears to be that the amount of agglutinin on the cell surface is affected both by the amount of agglutinin present and also by the ability of the cells to take up the agglutinin. It seems that the first factor determines the end point for cells with strong antigens and the second factor that for cells with weak antigens.

Table 47. Effect of Type of D Antigen on Titre
(After Renton and Hancock, 1958a)

Cells	*Titre of agglutinating anti-D serum Ho*										
	2	4	8	16	32	64	128	256	512	1,000	2,000
-D-/-D- ..	v	v	v	v	v	+++	++	++	+	−	−
Strong D	v	v	v	v	v	+++	++	++	+	w	−
R_2R_2 ..	v	v	v	v	v	+++	++	++	+	−	−
Normal D	v	v	v	v	+++	++	++	++	−	−	−
D^u HG ..	+++	+++	+++	++	+	−	−	−	−	−	−
D^u LG ..	++	+	w	−	−	−	−	−	−	−	−

HG = high grade. LG = low grade.

It is well known that antigen and antibody can combine together in varying proportions so that the amount of antibody combining with a fixed amount of antigen depends on the concentration of antibody present. Fig. 4 shows the main quantitative features of the reaction. When the amount of antibody in the serum is small most of it will be taken up by the cells, and as the amount of antibody is increased the amount taken up by the cells increases at first to almost the same extent. This is [illegible]

[illegible] shown by the top part of the curve. Now different antigens can take up different amounts of antibody from the same serum so that there is really a family of curves for the different D antigens each showing the same general features, but with a great deal more antibody needed to reach the top part of the curve in the case of the strong antigens than in the case of the weaker ones. With the stronger antigens, the amount of antibody needed at the cell surface for agglutination to take place lies on the lower part of the curve where the antibody taken up by the cells is almost equal to the antibody

are, and which types react in which tests, renders the whole subject rather confusing.

The first distinction which was drawn was between the saline agglutinin which agglutinates cells in saline suspension, and the incomplete antibody which fails to do so and is only detectable by other means. Even this first distinction, however, is not always as clear cut as at first sight seems to be the case, since some of the tests for incomplete antibody give positive results with agglutinin also, and since incomplete antibody has a blocking effect which may interfere with the agglutinating effect of the agglutinin, so that the fact that a serum fails to agglutinate cells in saline suspension does not mean that the serum is necessarily free from agglutinin.

Incomplete antibodies have themselves been thought to be non-homogeneous and, as a result of discrepancies between results given by different tests for incomplete antibody and by various physical and chemical fractionating procedures, incomplete antibodies have been divided into a number of types which have been referred to by terms such as "agglutinoid", "cryptagglutinoid", "blocking antibody" and "albumin-agglutinin". However, the complications which occur even in the case of the simple division into saline agglutinin and incomplete antibody have even more force when one attempts to subdivide incomplete antibody into different types. Another difficulty is that the tests vary in sensitivity so that some of the differences are of a purely quantitative nature. As a result of these difficulties, no clear picture of the different types of incomplete antibody which exist has emerged, though there is no doubt of the existence of more than one type.

The existence of various ill-defined types of incomplete antibody has, however, little practical importance, and, provided it is remembered that the different tests for incomplete antibodies do not always detect exactly the same thing, it can generally be ignored. The widespread use of saline agglutination techniques, on the other hand, means that the distinction between saline agglutinin and incomplete antibody is of considerable importance. These two types of antibody compete with one another for the antigen on the cell surface, and under some circumstances the presence of incomplete antibody can accordingly interfere with the action of the agglutinin. The extent of this interference is in turn influenced by the properties of the antigen involved and the [illegible]

anti-D with the different types of D antigen, but agglutinins and incomplete antibodies of other specificities are well known, and there is no reason to suppose that they behave differently from anti-D.

AGGLUTINABILITY OF CELLS WITH VARIOUS TYPES OF D ANTIGEN. If incomplete antibody is present when saline suspensions of cells are tested with anti-D sera, it can combine with some of the antigen on the red cell surface and thereby interfere with the uptake of agglutinin. An important result of this blocking effect is that it can mask the presence of agglutinin in anti-D sera. Now cells with various types of D antigen require various amounts of incomplete antibody for blocking to occur, and since anti-D sera vary in the proportion of *incomplete antibody* to agglutinin which they contain, it follows that cells of various types will be agglutinated in saline by a varying proportion of anti-D sera. It must be remembered that sera are classified as "agglutinating" or as "incomplete" sera according to their reactions with normal D cells in saline suspension, and that this is really an arbitrary distinction. Those sera which contain sufficient incomplete antibody to cause blocking with a certain strength of antigen will not agglutinate cells with weaker antigens since blocking will be even more marked. They may, however, agglutinate cells with stronger antigens. This is the reason for the [illegible] D [illegible] D [illegible] Strong D [illegible] c[illegible] D cells [illegible] a [illegible] incomplete antibody and an incomplete serum, and also that between an agglutinin and an agglutinating serum. An anti-D serum which contains a *sufficiently* low proportion of incomplete antibody to enable it to agglutinate cells of any given type will be found to agglutinate all cells with stronger D antigens. The pattern which results from this is *shown* in Table 48, where the results of testing fifty unselected anti-D sera with cells of different strengths of D antigen are shown.

TABLE 48. REACTIONS OF FIFTY ANTI-D SERA WITH SALINE SUSPENSIONS OF CELLS HAVING VARIOUS D ANTIGENS
(After Renton and Hancock, 1958a)

Number of sera	*Type of antigen*				
	-D-/-D-	*Strong D*	*Normal D*	*D^u HG*	*D^u LG*
2	+	+	+	+	+
3	+	+	+	+	−
17	+	+	+	−	−
*1	+	+	−	+	−
6	+	+	−	−	−
12	+	−	−	−	−
9	−	−	−	−	−

This table shows the number of sera giving each pattern of reaction.
+ = Positive reaction HG = high grade
− = Negative reaction LG = low grade
* = Atypical, but unfortunately could not be retested.

present. This means that the cells will agglutinate in a certain concentration of agglutinin irrespective of the strength of antigen at the cell surface, provided this is strong. The end point of the titration will thus only depend on the concentration of agglutinin present and will be independent of the antigen. With the weaker antigens, on the other hand, the amount of agglutinin on the cell surface needed for agglutination lies on the upper part of the curve, so that a high concentration of agglutinin is needed and the titre is thus reduced as compared with the stronger antigens. Furthermore, it can be shown that the cells with the weaker antigens need to take up more agglutinin for agglutination to occur than do cells with stronger antigens, as can be shown by the fact that D^u cells from the first negative tube of a titration will agglutinate when washed and mixed with R_2R_2 cells (Renton and Hancock, 1958b).

The consequence of these considerations is that antigen strength only affects the titre in the case of the weaker antigens.

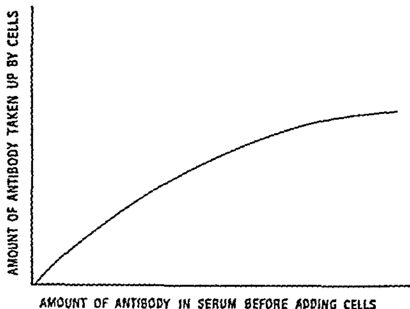


FIG. 4. Hypothetical curve of antigen-antibody reaction.

Effects of Interaction of Incomplete Antibody and Agglutinin

The effects produced by the interaction of incomplete antibody and agglutinin are of some practical importance, and one way out of the difficulty caused by ignorance of the true nature of the different types of incomplete antibody is to define incomplete antibody as "antibody which fails to agglutinate cells in saline suspension but which competes with the agglutinin for the available antigen sites". The advantage of this definition is that it embraces all types of incomplete antibody and so enables one to consider the various effects produced by the interaction of incomplete antibody and agglutinin.

serum. Table 49 shows three titrations of an anti-D serum showing a prozone with D positive cells. The titrations were carried out simul-

TABLE 49. EFFECT OF SPATIAL RELATIONSHIP OF ERYTHROCYTES ON PROZONE

Condition of erythrocytes during incubation period	*Titre of saline anti-D*						
	1	2	4	8	16	32	64
Sedimentation by gravity for 1½ hr. then centrifuged 1,000 r.p.m. for 2 min.	w	++	+++	+++	++	+	−
Centrifuged 1,000 r.p.m. for 2 min. then incubated 1½ hr. with cells packed at bottom of tube; then centrifuged 1,000 r.p m. for 2 min.	+++	v	+++	+++	+	+	−
Agitated intermittently for 1½ hr. then centrifuged 1,000 r.p m. for 2 min. ..	−	−	+	+++	++	+	−

taneously, and the only difference between them was that the tubes were treated differently. In the first titration they were allowed to sediment for one and a half hours at 37° C. The tubes in the second titration were centrifuged immediately after the cells and serum had been mixed and were then incubated for one and a half hours with the cells in close contact with one another at the bottom of the tube. In the third titration the tubes were tapped at intervals during the incubation period to prevent the cells from settling. All the tubes were centrifuged at the end of the period of incubation before reading the results. It can be seen from this experiment that close contact of the cells during the reaction promotes the second stage of the agglutination reaction so that zoning is diminished. Keeping the cells apart from one another whilst they are in the serum has the reverse effect. Normal D cells were used in this experiment and it is interesting to notice that the spatial relationship of the erythrocytes did not affect the titration end-point. Table 50 shows a similar experiment using D^u cells, and it can be seen that the titration end-point here is affected by the spatial relationship of the erythrocytes. We have seen above that the titration end-point is only

TABLE 50 EFFECT OF SPATIAL RELATIONSHIP OF ERYTHROCYTES ON THE END-POINT OF A TITRATION WITH D^u CELLS

Condition of erythrocytes during incubation period	*Titre of saline anti-D*						
	1	2	4	8	16	32	64
Sedimentation by gravity for 1½ hr. then centrifuged 1,000 r.p.m. for 2 min. ..	v	v	+++	+++	+	w	−
Agitated intermittently for 1½ hr. then centrifuged 1,000 r.p m. for 2 min. ..	+++	++	+	w	−	−	−

To cause agglutination a serum must have (i) a sufficient amount of agglutinin, and (ii) a sufficiently low proportion of incomplete antibody. For normal D, R_2R_2, Strong D and —D—/—D— cells, the amount of agglutinin required is practically the same, and the proportion of incomplete antibody is the determining factor. For D^u cells more agglutinin is needed than for D cells, and both factors are important.

ZONING. Zoning is a phenomenon in which, although no agglutination is observed with low dilutions of serum, agglutination does occur when the serum is diluted further. This phenomenon can, in theory at any rate, take place due to the presence of excessive amounts of agglutinin alone, but it is much increased by the presence of incomplete antibody. It is also dependent on antigen strength and is always more marked with a weaker antigen than with a strong one.

The basic reason for this phenomenon seems to be that agglutination takes place in two stages. In the first stage the cells take up molecules of agglutinin free in the serum, and in the second stage they take up molecules already attached to other cells, and agglutinate in consequence.

The two stages may well be concurrent. The second stage is favoured [illegible] the cell surface, since this reduces the ability of the cells to take up agglutinin already attached to another cell. It does not matter in this connection whether the cell has taken up agglutinin alone or incomplete antibody and agglutinin in the first stage of the reaction, since both will have the effect of reducing the ability of the cell to take up agglutinin in the second stage. A very high concentration of agglutinin alone could itself interfere with the second stage sufficiently to prevent agglutination, causing a prozone due to pure agglutinin excess. If incomplete antibody is present as well as agglutinin the same mechanism would appear to operate as in the case of agglutinin alone. The prozone in each case is due to excessive amounts of antibody at the cell surface interfering with the second stage of the reaction. With incomplete antibody present, however, zoning occurs much more readily than with agglutinin alone, since the former has only a hindering effect on the second stage whilst the latter has both a hindering and an en- [illegible] these two factors that determines the occurrence of agglutination (Renton and Hancock, 1958b).

The fact that agglutination takes place in two stages is illustrated by the fact that the length of the prozone is dependent on the spatial relationship of the erythrocytes whilst they are in contact with the

serum. Table 49 shows three titrations of an anti-D serum showing a prozone with D positive cells. The titrations were carried out simul-

TABLE 49. EFFECT OF SPATIAL RELATIONSHIP OF ERYTHROCYTES ON PROZONE

Condition of erythrocytes during incubation period	*Titre of saline anti-D*						
	1	2	4	8	16	32	64
Sedimentation by gravity for 1½ hr. then centrifuged 1,000 r.p.m. for 2 min.	w	++	+++	+++	++	+	−
Centrifuged 1,000 r.p.m. for 2 min. then incubated 1½ hr. with cells packed at bottom of tube; then centrifuged 1,000 r.p m. for 2 min.	+++	v	+++	+++	+	+	−
Agitated intermittently for 1½ hr. then centrifuged 1,000 r.p m. for 2 min. ..	−	−	+	+++	++	+	−

taneously, and the only difference between them was that the tubes were treated differently. In the first titration they were allowed to sediment for one and a half hours at 37° C. The tubes in the second titration were centrifuged immediately after the cells and serum had been mixed and were then incubated for one and a half hours with the cells in close contact with one another at the bottom of the tube. In the third titration the tubes were tapped at intervals during the incubation period to prevent the cells from settling. All the tubes were centrifuged at the end of the period of incubation before reading the results. It can be seen from this experiment that close contact of the cells during the reaction promotes the second stage of the agglutination reaction so that zoning is diminished. Keeping the cells apart from one another whilst they are in the serum has the reverse effect. Normal D cells were used in this experiment and it is interesting to notice that the spatial relationship of the erythrocytes did not affect the titration end-point. Table 50 shows a similar experiment using D^u cells, and it can be seen that the titration end-point here is affected by the spatial relationship of the erythrocytes. We have seen above that the titration end-point is only

TABLE 50. EFFECT OF SPATIAL RELATIONSHIP OF ERYTHROCYTES ON THE END-POINT OF A TITRATION WITH D^u CELLS

Condition of erythrocytes during incubation period	*Titre of saline anti-D*						
	1	2	4	8	16	32	64
Sedimentation by gravity for 1½ hr. then centrifuged 1,000 r.p.m. for 2 min. ..	v	v	+++	+++	+	w	−
Agitated intermittently for 1½ hr. then centrifuged 1,000 r.p.m. for 2 min. ..	+++	++	+	w	−	−	−

dependent on the antigen in the case of the D^u cells. This
further illustration of the same
spatial
in and it is
po. importance in the Sandwich test also.

EFFECT OF INCOMPLETE ANTIBODY ON AGGLUTININ TITRE. The presence of incomplete antibody in a saline agglutinating serum has no effect on its titre. The reason for this is that when the proportion of incomplete antibody in a serum or mixture of sera is
amount of agglutinin remains

the second stag though it is likely that a further increas proportion of incomplete antibody present would also cause a reduction in the amount of agglutinin taken up in the *first stage* of the reaction. This phenomenon is shown in Table 51. Once agglutination has failed due to antibody excess, reducing the

TABLE 51. EFFECT OF INCOMPLETE ANTIBODY ON AGGLUTINATION OF NORMAL D CELLS
(After Renton and Hancock, 1958a)

	Reciprocals of dilutions of agglutinating anti-D serum								
P	2	4	8	16	32	64	128	256	512
0 to 1	v	v	v	v	v	v	++	+	—
1 to 64	v	v	v	v	v	v	++	+	—
1 to 32	v	v	v	v	v	v	++	w	—
1 to 16	v	v	v	v	v	+++	++	+	—
1 to 8	v	v	v	v	v	+++	++	w	—
1 to 4	v	v	v	v	v	+++	++	+	—
1 to 2	v	v	v	v	v	+++	++	w	—
1 to 1	++	v	v	v	v	+++	++	+	—
2 to 1		+	+++	+++	v	+++	++	+	—
4 to 1			—	+	++	+++	+++	+	—
8 to 1				—	w	+	++	+	—
16 to 1					—	—	w	w	—
32 to 1						—	—	—	—
64 to 1							—	—	—

Each tube contained 1 volume diluted agglutinating serum, 1 volume diluted incomplete serum, and 2 volumes cell suspension.
Each column shows results for a constant *Dilution* of
Each row shows results for a
serum (e.g. for a tube co
serum diluted 1 in 16, P =

dilution of the serum will only serve to increase the antibody excess, so that agglutination cannot take place. The only way to get agglutination once it has been inhibited by antibody excess is to increase the proportion of agglutinin so that the agglutinin is able to overcome the antibody excess. It is the balance between the amount of agglutinin and the total amount of antibody which is the deciding factor.

Dosage

Some anti-Rh sera show the phenomenon of dosage; that is to say, higher titres or higher titration scores are seen when using cells from the homozygote than from the heterozygote. This is a constant and very marked feature of anti-c sera, and is well seen with many anti-E and anti-e sera also. The majority of anti-C sera show little or no dosage effect. With anti-D, not more than a trace of the effect is occasionally seen, and this is not only dependent on whether the individual is Dd or DD, but also appears to be dependent on the other Rh antigens and on other unknown factors also.

If the dosage effect is considered only as shown by the titre, then it can be seen that the considerations given above regarding the effect of antigen strength on titre are relevant. This means that differences of titre will only be seen in the case of antigens which are present on the red cell surface in small amounts. With antigens which are present in greater amounts dosage effects can sometimes be better demonstrated by comparing the titration scores. This may be due to incomplete antibody causing partial blocking of the weaker antigen of the heterozygous cells.

Effects of Interaction of Antibodies with Albumin Cell Suspensions

The use of 20 per cent bovine albumin cell suspensions shows the existence of different types of incomplete antibody, since antibodies

Table 52. Reactions of Anti-D Sera with Saline and 20 per cent. Bovine Albumin Suspensions of Cells having Various D Antigens

Serum No.	*Saline suspension of cells*					*Albumin suspension of cells*				
	-D-/-D-	*Strong D*	*Normal D*	*D^u HG*	*D^u LG*	*-D-/-D-*	*Strong D*	*Normal D*	*D^u HG*	*D^u LG*
30	v	v	+++	–	–	v	v	v	+++	–
21	++	w	–	–	–	v	v	v	++	–
27	+	–	–	–	–	v	v	v	++	–
25	++	–	–	–	–	v	v	+	–	–
15	+++	++	+	–	–	v	v	v	v	–
20	+++	+	+	–	–	v	v	+	–	–
11	v	+++	+	–	–	v	v	+++	–	–
18	–	–	–	–	–	v	v	v	+++	–

having an agglutinating effect in albumin and a blocking effect in albumin may be demonstrated. Furthermore, many of the effects caused by the interaction of saline agglutinin and incomplete antibody on saline suspensions of cells also occur through the interaction of the different types of antibody active against albumin suspensions of cells. These are illustrated by Table 52, which shows that those sera which have the least agglutinating action when tested against saline suspensions of cells having various types of D antigen are not necessarily the ones which give the weakest results against albumin suspended cells. This shows that part of the incomplete antibody has an agglutinating effect in albumin, and part of it has not, so that the albumin agglutinin acts as an incomplete antibody when the cells are tested in saline and as an agglutinin when they are tested in albumin.

Unusual Types of Rh Antibody

"Cold Anti-C." Rh antibodies not conforming to the usual pattern are occasionally seen. For example, we have seen several instances of "cold anti-C" in the sera of rr women. In saline, these acted predominantly in the cold, and sometimes, but not always, the reaction was enhanced by using serum albumin cell suspensions. Some of the antibodies failed to react by the indirect Coombs test. A dosage effect was shown by some of them; this is not often seen with anti-C sera.

Some of these sera had a strict anti-C specificity, others had a little anti-D also, and in one case a strong anti-D, acting best in the cold, developed later in pregnancy.

Some of the reactions of one of these "cold anti-C" sera are shown in Table 53.

Table 53. Reactions of a "Cold Anti-C" Serum, showing that it acts best at 16° C. and gives a Suggestion of Dosage

Cells		*Method of test*			
Name	*Group*	*Coombs test*	*Saline agglutination* 16° C.	37° C.	*Serum albumin* 37° C.
Fa	R_1R_1	—	v	—	+++
Pr	R_1R_1	—	v	—	+++
Ev	R_1r	—	++	—	w
Fi	R_1r	—	++	—	w
Bu	R_1R_2	—	+++	—	++
Sp	R'r	—	v	—	+++
Fr	R_2R_2	—	—	—	—
Ke	R_2r	—	—	—	—
Br	R_0r	—	—	—	—
Le	rr	—	—	—	—
Ke	rr	—	—	—	—

"PURE AGGLUTININS." Another unusual type of antibody is the "pure agglutinin" apparently free from incomplete antibody.

If the agglutinin is of moderate titre the absence of incomplete antibody is shown by the negative indirect Coombs test given by the serum (as in the case described below) but with a more potent agglutinin this effect is apt to be masked by the agglutination of the cells in the test before the Coombs reagent is added. In such cases, however, the strength of the result is the same whether saline or Coombs reagent is added in the test (see Table 54).

We have encountered three examples of this, all of specificity anti-D. The absence of incomplete antibody gave rise to two remarkable effects. Firstly, since it seems that agglutinin does not ordinarily pass the placental barrier, the antibody did not give rise to haemolytic

TABLE 54. TITRATION OF A "PURE AGGLUTININ" ANTI-D SERUM

	Titre of anti-D serum Ho							
	1	2	4	8	16	32	64	128
With saline added ..	++++	++++	++++	+++	++	+	–	–
With Coombs reagent added	++++	++++	++++	+++	++	+	–	–

Each dilution of the serum was tested by the Coombs technique in the usual way. After the sensitized cells had been washed they were divided into two portions, saline being added to one and Coombs reagent to the other. The fact that Coombs reagent does not enhance the titre suggests that little or no incomplete antibody is present, though a weak incomplete antibody would not be detected by this method.

disease of the newborn. Secondly, the sera agglutinated even low grade D^u cells in saline suspension. As discussed on p. 171, the inability of many agglutinating anti-D sera to agglutinate D^u cells is due to the blocking effect of the incomplete antibody which they contain. This effect does not obtain with a "pure agglutinin" and such sera can consequently agglutinate even low-grade D^u cells provided they are of sufficient titre. One such serum which we studied agglutinated thirty-five out of thirty-eight D^u bloods tested. Many of these were of low grade.

The reason for the absence of incomplete antibody in these sera is not known. A normal quantity of γ-globulin was found in the serum of one such patient (*vide infra*) and a second patient did develop incomplete antibody in a later pregnancy, as was shown by the fact that her D positive infant had a positive direct Coombs test. A previous D positive infant had had a negative direct Coombs test at birth.

The following case illustrates some of the features of these sera:

CASE 31

Mrs. E.D., a third para, had received a transfusion of two bottles of blood of unknown Rh type two years previously; she was group $AR''r$ (tests for D^u were negative), and her husband was group AR_1R_2. Antibodies were detected in her serum antenatally by the papain cell test and, on further investigation, gave the results shown below.

Further tests confirmed that the antibody was anti-D, and tests with different Coombs reagents were negative. The patient's serum contained a normal quantity of γ-globulin. D^u cells, even of low grade, were agglutinated in saline suspension by the patient's serum.

Mrs. E.D.'s baby was born by Caesarian section and had a haemoglobin of 17 gm. per cent; serum bilirubin 2·1 mgm. per cent at birth. It was not jaundiced and remained healthy. The baby was group AR_1R'' and no anti-D could be detected in its serum by the papain cell, Coombs or serum albumin techniques using R_1R_1 and R_2R_2 cells. The direct Coombs test was negative using two different

Cells		*Coombs test*	*Saline agglutination*		*Serum albumin*
			16° C.	*37° C.*	*37° C.*
	R_1R_1 ..	—	v	v	v
	R_1r ..	—	v	v	v
	R_2R_2 ..	—	+++	+++	+++
	R_2r ..	—	+++	v	v
Most known antigens	rr ..	—	—	—	—
	rr ..	—	—	—	—
	rr .	—	—	—	—

Coombs reagents, one of which was tested at nine different dilutions. Incubation with fresh adult serum did not cause the cells to give a positive direct Coombs test and they did not agglutinate when suspended in serum albumin, nor in fresh adult serum. No anti-D could be detected in an eluate of the child's cells. It was thus evident that the mother's anti-D had failed to pass through the placenta due to its being composed entirely of agglutinin.

The Preparation of Rh Typing Sera

THE PREPARATION OF ANTI-D TYPING SERA

ANTI-D SERA FOR THE TUBE TECHNIQUE. In most cases these sera come from Rh negative women who are mothers of infants suffering from haemolytic disease of the newborn. In general these and other anti-Rh sera should if possible be collected during the puerperium and the agglutinins are likely to be at their best between fifteen and twenty days post partum. *If it is not possible to obtain the patient's blood until* later it will sometimes be found that the antibodies have changed their properties *in vivo* and become less suitable for use as typing sera.

The patient is bled into a dry bottle and the serum separated in such a manner as to give a maximum yield (see p. 21). Preliminary tests are then carried out to see what antibodies the serum contains. We generally test the serum by Coombs, serum-albumin at 37° C., and saline agglutination at 16° C. and 37° C. with the following cells; R_1, R_2, R′, R″ and three or more selected rr cells bearing as many of the known blood group antigens as can be managed, so as to reveal the presence of antibodies of the other systems. These tests will usually give a pretty good idea as to what antibodies the serum contains, though further tests to identify these may be needed in some cases. An anti-D serum should give strong results in saline at 37° C. with the R_1 and R_2 cells and negative results with the other cells. Agglutination of the D positive cells at 16° C. is a favourable sign, usually indicative of good strength and avidity of the anti-D agglutinins. If antibodies other than anti-D are present these must be identified, but if they are not very strong it is likely that the serum does not need to be discarded, since they can often be removed during the absorption process. The serum must next be absorbed with A_1 rr or B rr cells or with both, as appropriate, in order to remove anti-A and anti-B. The technique described on p. 23 is suitable. When the absorption has been satisfactorily completed the serum should be titrated in saline with R_1 and R_2 cells at 37° C. and also retested with R′ and R″ cells. From the titration results one can see whether it will be possible to dilute the serum before use, but considerable discretion should be exercised, as it is very easy to be over-optimistic and dilute the serum too far. A diluent of normal AB serum tested with a variety of cells to exclude the presence of antibodies is better than saline. The titre of the serum is a very imperfect guide to its suitability as a reliable anti-D typing serum and the strength and firmness of the agglutination are often more valuable guides than the actual titration end-point. Sera which, though of high titre, give weak results all down the titration are unlikely to be satisfactory in use. The serum should still give at least a +++ result at a dilution eight times greater than that at which it is intended to be used, but in practice it is found that so great is the demand for serum, and so difficult is it to obtain a sufficient supply, that it is not always possible to keep to this standard. The final step before the serum is put into use is to test it in parallel with a serum of known reliability on two or three hundred samples of blood; if there are no discrepancies the serum may be used. This last step must not be omitted, since it is impossible to be certain by earlier tests whether it will in fact prove satisfactory.

It is not desirable to build up a large stock of anti-D typing serum absorbed and ready for use, since there is a considerable danger of anti-A and anti-B reappearing in the serum after absorption. The serum may change its properties on storage in other ways also, so that

the sera should only be processed a short time before they are to be used. The following example illustrates these procedures.

EXAMPLE OF THE TESTING AND PREPARATION OF ANTI-D SERUM FOR THE TUBE TECHNIQUE.

Serum. Mrs. M.P. group Orr.

Preliminary Tests

		Test		
Cells	*Coombs*	*Saline 20° C.*	*Saline 37° C.*	*Serum-Albumin 37° C.*
O R_1r	v	v	v	v
O R_2r	v	v	v	v
O rr MNS, P, kk, Lu(a−), Le(a+b−), Fy(a−), Jk(a+)	—	—	—	—
O rr Ms, p, kk, Lu(a+), Le(a−b+), Fy(a+), Jk(a+)	—	—	—	—
O rr NS, P, Kk, Lu(a−), Le(a−b+), Fy(a+), Jk(a−)	—	—	—	—

Preliminary Titration. Saline 37° C.

Cells		*Dilution, 1 in—* 1	2	4	8	16	32	64
O	R_1r	v	v	v	v	v	v	+++
O	R_2r	v	v	v	v	v	+++	+++
O	R′	+						
O	R″	—						

Remarks. A potent anti-D saline agglutinin with a trace of anti-C also. The titration has not been carried far enough, but this is not important because it is evident that this will be a useful serum in any case, and further titrations will be carried out after absorption.

Tests of absorbing cells

Cells	Anti- A	A_1	B	C+D	D	D	E
AT.7678	v	v	—	—	—	—	—
AT.7674	—		v	—	—	—	—

This confirms that the cells are A_1 rr and B rr and a mixture of equal parts of washed cells of the two types was used.

Tests on absorbed serum

Absorption test

Cells	*Type*	*16° C.*	*37° C.*
AT.7678	A_1rr	—	—
AT.7674	Brr	—	—

Titration. Saline 37° C.

Cells		*Dilution, 1 in—* 1	2	4	8	16	32	64	128	256	512	1,000
O	R_1r	v	v	v	v	v	+++	+++	++	+	—	—
O	R_2r	v	v	v	v	v	v	+++	+	+	w	—
O	R′	—	—	—	—	—	—	—	—	—	—	—
O	R″	—	—	—	—	—	—	—	—	—	—	—

Parallel tests. Tested at a dilution of 1 in 8 with 257 random bloods in parallel with the anti-D serum Bi and found to give good agglutinates and no discrepant results.

Conclusion. Suitable for use as an anti-D serum for the tube technique at a dilution of 1 in 8.

ANTI-D SERA FOR THE SANDWICH TECHNIQUE. These are prepared in a similar manner, and sera, which it is at first hoped will prove suitable for the tube technique, but which are subsequently found to have an insufficiently strong agglutinin to give reliable results, are usually suitable for this purpose. There is usually a plentiful supply of such sera. Sera for the Sandwich test ought to be tested with two or three hundred bloods of known Rh type by the Sandwich technique before they are put into use, and they should also be tested with Rh negative cells carrying as many as possible of the known blood group antigens by the same technique. The principle in all such cases is to test the serum by the technique for which it is intended, but this does not absolve one from the necessity of also testing the serum by the standard techniques. The reason for this is that contaminating antibodies may be present which will only react weakly by the Sandwich technique (or other special techniques) and which will perhaps react more strongly by one of the standard techniques. In most cases this means using the Coombs test, serum-albumin test and agglutination tests by the tube technique at 16° C. and 37° C. with cells bearing most of the known blood group antigens.

INCOMPLETE ANTI-D FOR THE DETECTION OF D^u. These should be as strong as possible and a watch should be kept for strong examples of incomplete anti-D from cases of Rh incompatible transfusion, and from mothers of cases of hydrops foetalis, stillbirths due to Rh incompatibility and the like, and supplies of serum obtained from the patients where possible. From such sera the best ones may be selected empirically by testing them in the Coombs test with low grade D^u cells and selecting those which give the best results. In any case it is essential that these sera shall be absolutely free from traces of anti-C and anti-E; the former is the commoner contaminant. To exclude anti-C, Coombs tests should be carried out with a number of examples of R′ blood, freshly collected if possible. Tests ought not to be confined to one or two examples of R′ blood only, since certain examples of R′ appear to contain an exceptionally strong C antigen reacting with traces of anti-C which are not apparent when other R′ cells are used (see p. 159). Tests with papainized R′ cells ought also to be included. Traces of anti-D agglutinin do not impair the use of the serum for the detection of D^u.

ANTIGLOBULIN TEST CONTROL SERUM. This is an incomplete anti-

D used to prepare sensitized cells for use as a control in the Coombs test. It ought to be completely free from agglutinin, but traces of incomplete anti-C are not important; the serum need not necessarily be absorbed since one can always use group O cells in the preparation of the control. It is important to have this serum of the correct strength, diluting it with serum if necessary. If the control cells are too strongly sensitized they will still be agglutinable by weak Coombs reagent or by Coombs reagents which have been partially neutralized by accidental contamination with human serum so that the control, which is a control of the washing of the cells in the Coombs test and of the fact that the Coombs reagent is working satisfactorily, will be insensitive. On the other hand, it is undesirable to have the Coombs control serum too weak, since minor variations in the preparation of the sensitized cells from day to day will sometimes result in failure to achieve detectable sensitization. The standard to be aimed at with this serum is that the sensitized cells prepared by its use will give a +++ result after six minutes with the routine Coombs reagent.

PREPARATION OF OTHER ANTI-Rh TYPING SERA

There is little that needs to be said about this, since the methods used are much the same as for anti-D and the main difficulty in most cases is to secure suitable sera to start with. Saline agglutinating sera are not always available and in this case the sera will have to be tested and used by whatever method they are found to react best.

ANTI-C. Anti-C sera usually present the greatest difficulty and, owing to the great rarity of pure anti-C in Rh positive people, agglutinating anti-C+incomplete anti-D sera have to be used as saline agglutinating anti-C sera. It is necessary to show that such sera give a positive result with the weakest C antigens likely to be encountered, and a negative result with the strongest D antigens likely to be encountered. The C antigen in R_1r cells reacts more weakly than that in R'r cells and R_1R_2 cells react more weakly still. The latter should, therefore, be used as the test cells. The strongest D antigens commonly encountered occur in R_2R_2 cells and these should always be used to test anti-C sera for freedom from anti-D agglutinin. Even so, the serum will still be liable to give false positive results with Strong D or -D- cells. It is, however, scarcely practicable to prepare anti-C sera which will not do this. Sera are sometimes encountered which contain a good saline agglutinating anti-C, unfortunately marred by the presence of a weak anti-D agglutinin. The problem then is to get rid of the anti-D agglutinin. Sometimes this can be achieved by simply diluting the serum and at other times by adding to it sufficient incomplete anti-D to block the action of the anti-D agglutinin. Incomplete anti-D sera vary widely in their blocking effect depending on the strength of incomplete antibody

present, and on the amount of concealed agglutinin present. It is probable that most, if not all, incomplete anti-D sera contain anti-D agglutinin even though this is masked by the blocking effect of the incomplete antibody when the sera are tested with normal D cells. In view of this it is best to select suitable blocking sera by empirical means, i.e. by testing a number of strong incomplete anti-D sera for their blocking effect against a weak anti-D agglutinin, and selecting those which give the best results. The correct proportion of incomplete anti-D serum to add to the anti-C serum should then be determined by trial and error, adding if possible rather more than the minimum amount actually required.

Removal of the anti-D agglutinin by absorption with R_2 cells is less often successful. There are two reasons for this. In the first place, most of the absorptive action of the R_2 cells often has to be expended in removing a strong incomplete anti-D which is also present in the anti-C serum so that even though there may only be a little anti-D agglutinin evident in the serum it is often very difficult to remove by absorption. In the second place, different anti-C+D sera vary in the extent to which it is possible to separate the two antibodies by absorption, so that in many cases removal of the anti-D by absorption will weaken or remove the anti-C also.

ANTI-E, c AND e. Anti-E sera do not usually present much difficulty, although if they are anti-E+incomplete anti-D sera the problems are similar to those which obtain in the case of anti-C+incomplete anti-D sera. The most suitable test cells in this case would be R_1R_2 as positive test cells, and R_1R_1 as negative test cells. Anti-E sera from Rh positive persons may be contaminated with anti-c. Similarly anti-c and anti-e sera may be contaminated with anti-E and anti-C, also with anti-f (this has been discussed on p. 157). Anti-c and anti-e always, and anti-E frequently, show dosage effects. That is to say they react much more weakly with cells which are heterozygous for the correspond[illegible]
such a char[illegible]
to their ider[illegible]
tested with homozygous cells they may fail to give reliable results with heterozygous cells. The correct test cells to use are R_1r for anti-c, R_2r for anti-e, and R_1R_2 for anti-E. There is sometimes difficulty in finding suitable cells for the absorption of these sera; a difficulty which may be illustrated by considering as an example an anti-e serum of group O. Such a serum requires absorption with either A_1B R_2R_2 cells, or with both $A_1R_2R_2$ and B R_2R_2. Now A_1B R_2R_2 cells can only be detected by the use of an absorbed anti-e serum, which is the very reagent which we are trying to produce, so that unless one already has such a serum it would seem that an impasse has been reached. There

are two possible ways out of the difficulty. One is to carry out titrations with a number of A_1B, E positive cells, using an anti-E serum known to give good dosage effects. If a blood is found which gives a markedly higher titration score than the others with the anti-E serum, it is reasonable to assume that it is most probably R_2R_2. A trial absorption of the anti-e serum could then be carried out using this blood and if this proved satisfactory it could be used to absorb the bulk. A second way out of the difficulty is by taking advantage of the different thermal optima of the antibodies concerned. Anti-A and anti-B act better in the cold than at 37° C. whilst Rh antibodies almost always act best at 37° C. and are frequently inactive at 16° C. It is thus possible to add A and B secretor saliva to the unabsorbed serum in order to use the mixture for testing A_1B R_2 bloods to find an A_1B R_2R_2. Before using the mixture for this purpose it should be tested and should give the results shown below:

Cells	A_1B	OR_2r	Orr
Temperature	16° C.	37° C.	16° C.
Results	Neg.	Pos.	Neg.

The negative result with Orr at 16° C. shows that the anti-e in the mixture is not active at this temperature, so that any agglutination obtained with AB cells can *only* be due to anti-A and anti-B. The positive result with OR_2r at 37° C. shows that the anti-e is active at this temperature after the addition of saliva and thus can be used for the purpose for which it is intended. The negative result when the mixture is tested with A_1B cells at 16° C. shows that the addition of the saliva has been sufficient to neutralize the anti-A and anti-B in the serum, and if these are inactive at 16° C. it may be safely assumed that they are still inactive at 37° C. This manoeuvre should enable one to select a suitable absorbing cell, and the principles involved are, of course, applicable to other antisera also. In the tests shown above it should be noticed that the Rh type of the A_1B cells used is immaterial. R_2r cells are used in the test at 37° C. because these contain the weakest e antigen likely to be encountered. The more strongly reacting rr cells are used in the test at 16° C., however, so as to show that the antibody is not active at all at this temperature.

Methods of Rh Typing

A great variety of methods of Rh typing have been described by various authors; some of these appear to be reliable while others are not. We have not, of course, had extensive experience of all these methods, and shall therefore confine ourselves to mentioning three methods which have given consistently good results in our own hands.

THE TUBE TECHNIQUE. One vol. (0 04 ml.) of a 3 per cent suspension of the cells to be tested is placed in a precipitin tube with one vol.

f saline agglutinating anti-D serum; the contents of the tube are [illegible]
y tapping the tube and the tube incubated for an hour and a half to two
ours at 37° C. The result is then read by pipetting the contents of
he tube on to a microscope slide and examining microscopically. This
s the most reliable method of all, its main disadvantage being that it is
rather slow. This is sometimes an important consideration in the D
typing of patients for transfusion.

THE CENTRIFUGE TECHNIQUE. This test is carried out in the same way as the tube technique except that the tubes are centrifuged at low speed after ten to fifteen minutes incubation and then read. It is desirable to carry out the incubation in a water bath, since an incubator may not provide a sufficiently rapid warming of the tube. The advantage of this method, of course, is its rapidity. The disadvantages are, firstly, that it is rather insensitive and will only give satisfactory results with the more potent saline agglutinating anti-D sera, and secondly, the centrifugation makes the test rather more difficult to read. It is very important in carrying out this test to centrifuge for the correct time at the correct speed. The cells should all be firmly packed to the bottom of the tube at the end of the period of centrifugation, but excessive centrifugation will lead to "comet" formation and make the test very difficult to read. The optimal degree of centrifugation is that which gives rise to a trace only of "comet" formation. We have found two to three minutes at 500 to 1,000 r.p.m. satisfactory.

THE SANDWICH TECHNIQUE (Stratton, 1955). One vol. (0·03 ml.) of a suitable anti-D serum (p. 181), one vol. of 30 per cent bovine albumin and one vol. of once washed packed cells are placed on a microscope slide. All the reagents are mixed together and a second microscope slide placed on the top so as to form a sandwich. Any excess fluid exuding from the side may be wiped away with a cloth. The "sandwiches" are then placed in a 37° C. incubator and may be read by microscopic examination ten minutes later. A moving field may be examined by pressing with the finger on top of the "sandwich" whilst it is being examined microscopically. The advantages of this method are speed, accuracy, and the ability to give good results with poor sera. A thousand bloods of known Rh group were tested by this method. There were no false positive results and only four false negative results, all of which were due to the presence of the D^u antigen. It is a test which is mainly useful in the rapid determination of the Rh type of patients prior to transfusion.

In the case of anti-Rh sera other than anti-D the methods used have to be adapted to the activity of the particular serum concerned, and the serum used by whichever method it works best; thus tests have to be

are two possible ways out of the difficulty. One is to carry out titrations with a number of A_1B, E positive cells, using an anti-E serum known to give good dosage effects. If a blood is found which gives a markedly higher titration score than the others with the anti-E serum, it is reasonable to assume that it is most probably R_2R_2. A trial absorption of the anti-e serum could then be carried out using this blood and if this proved satisfactory it could be used to absorb the bulk. A second way out of the difficulty is by taking advantage of the different thermal optima of the antibodies concerned. Anti-A and anti-B act better in the cold than at 37° C. whilst Rh antibodies almost always act best at 37° C. and are frequently inactive at 16° C. It is thus possible to add A and B secretor saliva to the unabsorbed serum in order to use the mixture for testing A_1B R_2 bloods to find an A_1B R_2R_2. Before using the mixture for this purpose it should be tested and should give the results shown below:

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THE TUBE TECHNIQUE. One vol. (0·04 ml.) of a 3 per cent suspension of the cells to be tested is placed in a precipitin tube with one vol.

be neglected. In the sandwich technique rouleaux are apt to be troublesome at times, and an AB serum control is essential here.

THE Rh TESTING OF DONORS

Only rr blood is suitable for transfusion to D negative recipients so that donors have to be tested with anti-C and anti-E as well as with anti-D. The reason for this is not that the C and E antigens are particularly antigenic (otherwise it would be necessary to avoid transfusing R_1 patients with R_2 blood and vice versa). The reason is that D negative patients are always liable to form anti-D in their serum and where anti-D is formed anti-C, and to a lesser extent anti-E, are likely to be formed also, so that if such patients are transfused with R′ or R″ blood a haemolytic transfusion reaction would result. Anti-C+D and anti-D+E sera can, of course, be used for this purpose. A further reason for testing donors with anti-C and anti-E is that this excludes most of the D^u bloods which have been overlooked in the tests with anti-D. The reason for this is that most D^u bloods are either R_1^u or R_2^u, which appear as R′ and R″ respectively if the D antigen is missed. R_0^u bloods will of course still be overlooked. These comprise only about 0·07–0·5 per cent of the apparently rr bloods in the British population. It is also advisable in Rh typing donors to use two different anti-D sera so as to reduce the chance of error. It is a debatable point whether all apparently rr donors ought to be tested with incomplete anti-D by the Coombs technique in order to detect R_0^u individuals, but in view of the low frequency of this type of blood in the white population, we ourselves do not believe that it is necessary to do this. In dealing with Negro or other populations, having a high incidence of R_0 and R_0^u, however, there is no doubt that it is essential.

The Detection of Rh Antibodies

The methods of detecting Rh antibodies most usually employed are the Coombs test, the 20 per cent bovine albumin test, the serum albumin test and the papain cell test.

Of these tests there is no doubt that the papain cell test is the most sensitive and it will detect Rh antibodies of all types and of all specificities, including some which fail to react in the other tests. A further advantage is that it will often detect antibodies earlier in pregnancy than the other tests. We have never detected an Rh antibody by the Coombs or albumin tests which did not also react by the papain cell test.

The Coombs test is the second best of these tests but fails to detect 6·4 per cent of Rh antibodies (see Table 55). It differs from the papain cell test mainly in being less sensitive, so that most of the antibodies which are Coombs negative and papain positive are only of low titre.

carried out by the serum albumin technique, by the Coombs technique, and even on occasions by papainizing the cells to be tested.

CONTROLS

The use of suitable controls in Rh typing is an important but much neglected matter. Two types of control are needed, those which control the activity and specificity of the testing serum, and those which control the cell suspension under test.

CONTROLS OF SERUM. The first of these is an absorption control to show that the serum is free from anti-A and anti-B. This is necessary [illegible] in sera from which they [illegible] For this reason the serum [illegible] better to do this control at 16° C. rather than at 37° C. since there is always a variable degree of cooling in reading the results of Rh typing, and if the test was cooled more than the control (the latter being carried out at 37° C.) it might happen that a trace of anti-A which remained undetected by the control would give a false positive result in the test. If the control is done at 37° C., then the test ought to be read first and the control last. Whenever possible these controls ought to be put up with each batch of tests, but if there is difficulty in obtaining suitable control cells it would probably be sufficient to carry out the test at, say, weekly intervals.

The other controls of the serum needed are a positive control to show that the anti-D agglutinin is active (ideally an R_1r cell should be used but in most cases any D positive cell would be suitable), and a negative control to show that the serum is not agglutinating all cells irrespective of their Rh type. The necessity for these two controls again depends upon the circumstances. If a large number of tests are carried out simultaneously, positive and negative results will be encountered and these will serve to control one another so that no additional control test would be needed, but if only one test is carried out at a time at infrequent intervals the control should be carried out at intervals, or whenever doubt arises. If working with an unfamiliar serum one should always include controls. With the other anti-Rh sera, special control cells are needed and these are the same as the cells required to establish the specificity of the sera, as described above.

CONTROL OF CELL SUSPENSION. There is always a possibility that the cells tested may be affected by autoagglutinins, rouleaux, infection, or some of the other causes of false positive results, and it is very necessary to put up a control to show that this had not occurred. A vol. of cell suspension should, accordingly, be tested with a vol. of saline, or better still with a vol. of AB serum, at the same time as the test is performed. This is a most important control which ought never to

R_1R_2 is unsatisfactory owing to the poor reactivity of the C and E antigens. The R_1 cells may be R_1R_1 or R_1r, though the use of the former confers but little advantage in the detection of anti-C or anti-D. R_2R_2 cells, on the other hand, are better than R_2r for both anti-D and anti-E.

Since both R_1 and R_2 cells have to be used, the question arises as to whether to use them separately and thus double the number of tests needed or whether to use a mixture. We think it better to use them separately, though it is less unsatisfactory to use a mixture in the papain and Coombs tests than in the serum/albumin or saline agglutination tests. The reason for this is that if the antibody present reacts with only one component of the mixture (anti-E for example), then the non-reacting (R_1) cells tend to interfere with the agglutination of the reacting (R_2) cells. In the papain and Coombs tests, however, the rocking motion on the slide assists one reacting cell to come into contact with another so that the interference is less marked. Indeed in all tests on cell mixtures a method which includes rocking on a slide is advantageous, and if only a few reacting cells are present, then the advantage may be considerable.

References

Callender, Sheila T., and Race, R. R. (1946). A serological and genetical study of multiple antibodies formed in response to blood transfusion by [illegible]

Cep[illegible], 13, 102.

[illegible] A new allele [illegible]

Ceppellini, R. (1950). L'Antigène Rh E^u. *Rev. d'Hém.*, 5, 285.

De Natale, A., Cahan, A., Jack, J. A., Race, R. R., and Sanger, Ruth (1953). V, a "new" Rh antigen, common in negroes, rare in white people. *J. Amer. Med. Assoc.*, 159, 247.

Giblett, Eloise R., Chase, J., Motulsky, A. G. (1957). Studies on anti-V, a recently discovered Rh antibody. *J. Lab. and Clin. Med.*, 49, 433.

Greenwalt, T. J., and Sanger, Ruth (1955). The Rh antigen E^w. *Brit. J. Haem.*, 1, 52.

Plaut, [illegible]

Ra[illegible]

Race, R. R., Sanger, Ruth, and Lawler, Sylvia D. (1948b). Rh genes allelomorphic to D. *Nature*, 162, 292.

Race, R. R., Sanger, Ruth, and Lawler, Sylvia D. (1948c). The Rh antigen D^u. *Ann. Eugen.*, 14, 171.

Race, R. R., Sanger, Ruth, and Selwyn, J. G. (1950). A probable deletion in a human Rh chromosome. *Nature*, 166, 520.

TABLE 55. RESULTS OF TESTING SERA OF RH NEGATIVE ANTENATAL PATIENTS BY THE PAPAIN AND COOMBS TESTS

Total tests = 18,209
Number of anti-Rh detected = 1,000 (5 49%)

Papain positive : Coombs test positive	=	936 (93 6%)
Papain positive ; Coombs test negative	=	64 (6 4%)
Papain negative : Coombs test positive	=	0

Of the two albumin tests, the serum albumin test is better than the 20 per cent bovine albumin test, though both are less satisfactory than the Coombs and papain tests. Strong Rh antibodies which are Coombs positive and albumin negative are not infrequent. The converse, an Rh antibody which is Coombs negative and albumin positive, is unusual, but it does occur. In most cases this is due to the antibody being predominantly or entirely saline agglutinin, but Rh antibodies which are negative in saline and in the Coombs test, but are positive in the albumin tests, are also seen, though these are very rare. The saline agglutinin does not really react in the Coombs test though it will, of course, act in the albumin tests. If it is strong, the cells in the Coombs test will agglutinate and a positive result will be recorded, though an equally strong result would be obtained without the use of Coombs reagent. If the agglutinin is weak, however, it will probably be overlooked in this test since the agglutinates will be broken up in the washing process.

All these tests are to some extent delicate and liable to technical error, and it is therefore desirable to use two different tests for the detection of Rh antibodies. The papain cell test and the Coombs test would seem the best choice.

Whatever test one uses the question of the type of cells to use requires consideration, since the antigens of cells of certain types react more strongly than those of others. The most strongly reacting type of cell should be used.

For anti-D: R_2R_2 cells are the best, but cells of other D positive types are almost equally good, and there appears to be nothing to be gained by using —D—/—D— or Strong D cells, even if these are available.
For anti-C: R'r cells, or better still R'R' if available, should be used. R_1R_1 and R_1r are less effective and R_1R_2 are worse still.
For anti-E: The situation is the same as for anti-C; R"r or better still R"R" if available, R_2R_2 and R_2r are less effective, and R_1R_2 give poor results.
For anti-c, anti-e and anti-f: rr cells should be used.

For the detection of Rh antibodies in general, when one does not know which antibody to expect, both R_1 and R_2 cells should be used.

CHAPTER IX

THE ORGANIZATION OF AN ANTENATAL TESTING SERVICE

IN this chapter we shall discuss the objects of an antenatal testing service and the means by which these objects may be achieved, and we shall describe the methods used in our own laboratory, and the way in which the work is organized. No doubt equally good results could be obtained if the work were organized in some other way, but the system described here has been in operation for a number of years and will, we hope, serve to illustrate some of the points which ought to be considered in deciding what system should be operated in an antenatal testing laboratory.

The primary object of the antenatal testing service is the identification during the antenatal period of those women whose infants are likely to be affected by haemolytic disease. In this way arrangements may be made for them to be delivered in hospital, for premature induction of labour, where considered desirable, and for suitable testing and treatment of the infant when it is born.

Subsidiary objects of the service are, firstly, to detect and identify antibodies present in the sera of antenatal patients so as to be prepared in case the mother, herself, requires transfusion at the time of delivery; and secondly, to acquire information about the antibodies and their clinical and serological behaviour, and thus to increase knowledge of these matters.

Since the vast majority of cases of haemolytic disease of the newborn are due to anti-D it is desirable to determine the D type of all antenatal patients; those who are D negative can then have their sera tested for the presence of antibodies during pregnancy. Since haemolytic disease of the newborn also occurs occasionally due to the other Rh antibodies and to antibodies of other blood group systems, it is not sufficient to confine the tests to D negative women.

If, however, it is not possible to carry out antibody tests on the sera of all D positive antenatal patients, a compromise has to be made, and the tests confined to the sera of those D positive patients who give a history suggestive of immunization or of haemolytic disease of the newborn. This is the procedure adopted in our own laboratory. Whilst the preliminary D-typing can often be carried out conveniently in hospital laboratories, the subsequent detection and identification of antibodies is a rather more complicated procedure, best performed in the laboratory of a blood transfusion centre or some similar specialized laboratory. It is with the procedures of such a laboratory that we are here concerned.

Such a laboratory receives a large number of samples of blood, some

Race, R. R., Sanger, Ruth, and Selwyn, J. G. (1951). A possible deletion in a human Rh chromosome: a serological and genetical study. *Brit. J. Exp. Path.*, **32**, 124.

Race, R. R., and Sanger, Ruth (1951). The Rh antigen C^u. *Heredity*, **5**, 285.

Renton, P. H. (1949). The Rhesus factor D^u. M.D. Thesis: Univ. of Manchester.

Renton, P. H., and Stratton, F. (1950). Rhesus type D^u. *Ann. Eugen.*, **15**, 189.

Renton, P. H., and Hancock, Jeanne A. (1955). An individual of unusual Rh type. *Vox Sang.*, **5**, 135.

Renton, P. H., and Hancock, Jeanne A. (1956). Variability of the Rhesus antigen D. *Brit. J. Haem.*, **2**, 295.

Renton, P. H., and Hancock, Jeanne A. (1958a). The influence of antigen strength, agglutinin, and incomplete antibody on agglutination by anti-D sera. *Brit. J. Haem.*, **4**, 89.

Renton, P. H., and Hancock, Jeanne A. (1958b). Rh antibodies and the prozone phenomenon. *J. Clin. Path.*, **11**, 49.

Rosenfield, R. E., Vogel, P., Gibbel, Natalie, Sanger, Ruth and Race, R. R., (1953). A "new" Rh antibody, anti-f. *Brit. Med. J.*, i, 975.

Rosenfield, R. E., Haber, Gladys, and Gibbel, Natalie (1957). A new Rh variant. *Proc. of the 6th Cong. of the Internat. Soc. of Blood Trans.*

Sanger, Ruth, Race, R. R., Rosenfield, R. E., Vogel, P., and Gibbel, Natalie (1953). Anti-f and the "new" Rh antigen it defines. *Proc. Nat. Acad. Sci. Wash.*, **39**, 824.

Stratton, F. (1946). A new Rh allelomorph. *Nature*, **158**, 25.

Stratton, F., and Renton, P. H. (1948). Rh genes allelomorphic to D. *Nature*, **162**, 293.

Stratton, F., and Renton, P. H. (1949). The Rhesus factor D^u. *Brit. Med. J.*, ii, 682.

Stratton, F., and Renton, P. H. (1954). Haemolytic disease of the newborn caused by a new Rh antibody, anti-C^x. *Brit. Med. J.*, i, 962.

Stratton, F. (1955). Rapid Rh typing, a "sandwich" technique. *Brit. Med J.*, i, 201.

Waller, R. K., Sanger, Ruth, and Bobbit, O. B (1955). Two examples of the —D—/—D— genotype in an American family. *Brit. Med. J.*, i, 198.

Wiener, A. S., Geiger, J., and Gordon, Eve B. (1957). Mosaic nature of the Rh_0 factor of human blood. *Exp. Med. and Surg.*, **15**, 75.

enables antibodies to be detected in a number of cases where they were not present at the beginning of pregnancy. Whether or not the test at 28 weeks is carried out, it is essential, if antibodies have not been detected already, to carry out a final test late in pregnancy. The later this can be done the better so as to reduce the chance of missing antibodies which may develop subsequent to the final test. If it is left too late, however, there is a danger of the women being delivered prematurely, before the test has been done, and also a danger that the opportunity may be missed for the premature induction of labour, if this is considered desirable. A test at 36 weeks would seem to offer the best compromise. We have seen a number of cases where antibodies developed late in pregnancy and where the child was severely affected with haemolytic disease of the newborn. A test at 36 weeks is essential. If no antibodies are found in early pregnancy it does not necessarily mean that the child will not suffer from [illegible]

de [illegible]

CASE 32

Mrs. A.O., group Orr, had had one normal child and no history of blood transfusions. No antibodies were detectable in her serum by the papain cell or Coombs test at 14 or 35 weeks, of her second pregnancy. When her infant was born it was found to be group O, R_1r, and the direct Coombs test was strongly positive. It was clinically normal and its haemoglobin level was within normal limits, but at the age of 20 days the infant was found to be jaundiced, its haemoglobin was only 5·7 grammes per cent and it had to be transfused with Rh-neg. blood. Tests of the mother's serum at this time showed a strong anti-[illegible] cells and detected by the C[illegible] techniques. A sample of h[illegible] preserved at −20° C. Th[illegible] antibody at this time confirmed.

The System

We shall now describe the system in use in our own laboratory. The first essential is an efficient card index system showing the results of all previous tests done on the patients. This is operated by a clerk who receives the samples of blood from hospitals and clinics, checks the previous records, resolves by phoning the hospital or clinic concerned any doubts which may arise as to the correct identity of the patient, and then proceeds to sort the specimens and to classify them according to their nature. Each specimen is allotted a code number, according to its type, as shown in the first column of Table 56. A laboratory work sheet is then made out showing the name and serial number of each specimen. Specimens of the same code number are listed together on

already D-typed, some already known to have antibodies, some from husbands and some from infants, and some on which no previous tests have been carried out, and it is very necessary to have a system, or scheme of operation, which will ensure that the right tests are carried out on the right samples. Whilst many samples can be adequately dealt with according to a fixed routine, there are others which require individual attention from a senior person, either in order to resolve discrepancies in the previous tests, or because some specialized test is called for. One of the main desiderata of the system is that it should be so arranged that the first type of specimen can be dealt with by junior staff, whilst the second type of sample is brought to the notice of the senior staff at the earliest possible stage. The system should also be designed so as to eliminate unnecessary repetition of tests and so as to ensure, so far as is practicable, that antibodies of all specificities are detected.

The Samples

A number of precautions which have to be observed in the collection of samples of blood for blood grouping work have already been discussed on p. 18. Clotted venous samples are the most suitable, although in occasional instances ABO and Rh grouping tests and direct Coombs tests can be carried out on citrated capillary samples. In the case of women with suspicious medical histories, or women with antibodies where extensive investigation may be needed to identify the antibody, it is important to have a sample large enough for the completion of all the necessary tests; 20 ml. of clotted blood is suitable. The question as to what period of pregnancy is best for the examination of the mother's blood for the presence of antibodies is one to which no accurate answer can be given. Antibodies may appear at any time during pregnancy and may be dangerous to the infant even though they do not appear until late in pregnancy. Sometimes antibodies are not detectable at any time during pregnancy but are present in the puerperium, so that the later in pregnancy one can do the tests the less likely it is that cases with antibodies present will be overlooked. On the other hand it is convenient, if antibodies are present, to know about this as early in pregnancy as possible so as to allow plenty of time to arrange confinement in hospital and so that a careful watch can be kept on the progress of the mother and foetus (e.g., it has been suggested that women with Rh antibodies are more susceptible to toxaemia). It is therefore best to determine the mother's Rh group and test for antibodies on the first occasion when she is seen by the doctor following the diagnosis of pregnancy, and a high proportion of antibodies are detected at this stage. Those who are D positive are not, in the absence of abnormalities of the history, tested further in our scheme, but those who are D negative require further tests. A second test for antibodies at 28 weeks of gestation, or thereabouts, is a useful though not essential step, which

TABLE 57. ACTION TO BE TAKEN ON COMPLETION OF ROUTINE ANTENATAL TESTS

Result of tests of maternal blood	*History*	*Action*
D positive .. .. .. D negative (no antibodies) ..	Normal Normal	Discard specimen and despatch report.
D positive .. D negative (no antibodies) ..	Abnormal Abnormal	For further investigation.
D negative (antibodies) ..		i. Further tests to identify antibody if needed. ii. Telephone results if patient is near to term. iii. Arrange for patient to be bled in due course if suitable for typing serum.

this sheet. The sheet has printed columns for recording the result of the appropriate test. The specimens are placed in a special rack in the same order as they appear on the laboratory worksheet. This procedure, together with Table 56, makes it easy to see which tests have to be carried out on which samples. When the tests shown in this Table are completed, the specimens are again sorted and dealt with according to Table 57. Further tests will be required in some cases to identify unusual antibodies, and all cases showing unusual features or discrepant results should also be set aside at this stage for further investigation.

The Tests

The tests enumerated in Table 56 comprise tests for erythrocyte antigens, antibody detection tests, antibody identification tests and additional tests on the blood of newborn infants. The techniques by which these tests are carried out are discussed in other chapters, but some of the considerations which apply to the application of these tests in an antenatal testing service will now be considered.

DETECTION OF ERYTHROCYTE ANTIGENS

THE ABO GROUPS. These should be determined by the tube technique, reading microscopically, but anti-A_1 is used by the slide technique. We are not in favour of issuing any written reports on the ABO group of antenatal patients, owing to the grave dangers of clerical errors, incorrect identification of the patient, and the like. Once such a report is issued there is always the danger that someone will transfuse the patient with blood of the same ABO group as indicated on the report without carrying out adequate cross-matching tests. The ABO group we feel should always be determined as part

TABLE 56. A SYSTEM FOR ANTENATAL TESTING

Code No.	Type of specimen	Routine Rh section	Routine antibody section	Special Rh section
1 2 3 4	Mothers never previously tested Mothers with no antibodies, not previously tested this pregnancy Repeat specimens from D pos. mothers who have been tested this pregnancy Specimens found D neg. in hospital laboratories	i. Screen with one anti-D (saline control)* ii. Second anti-D if neg. or weak (also all No. 4s) iii. ABO group if D neg (Anti-A_1 if appropriate)	Coombs and Papain tests if D neg.	If antibodies present: i. Test with anti-C and anti-E and for D^u if positive with either.‡ ii. Full antibody investigation (see 7).
		Special Rh section		
5	Repeat specimens from women found D neg. this pregnancy (no antibodies)	Coombs and Papain tests		
6	Women with antibodies (first specimen this pregnancy)	i. ABO group (Anti-A_1 if appropriate), two anti-D sera, anti-C and anti-E (test for D^u‡ if positive with either). ii. Papain test. iii Coombs test, serum albumin, saline agglutination tests at 16° C. and 37° C. with R_1, R_2, R', R'' and three or more selected rr bloods. iv. Such further tests as may be needed to identify the antibody. v. Preserve any remaining serum.		
7	Women with antibodies (previously tested this pregnancy)	Tests ii, iii, iv and v of preceding section.		
8	Husbands	ABO group (Anti-A_1, if appropriate), two anti-D sera, anti-C, anti-E, anti-c, anti-e (if appropriate and available) (saline control).* Test for D^u if positive with anti-C or anti-E.‡		
9	Babies of women known to have antibodies	i. Direct Coombs test. ii. ABO group,† two anti-D sera, anti-C, anti-E (saline control).*		
10	Other babies	i. Direct Coombs test. ii. ABO group.† Two anti-D sera (saline control).*		
11	Special Investigation: cases sent in specially, cases where discrepant results were obtained previously, D pos. women with antibodies, antibodies not of Rh system, husband and wife for joint investigation, etc.	Test according to circumstances.		

* Or better, AB serum control.
† Anti-A_1 gives unreliable results with the cells of newborn infants, and is therefore not used.
‡ The D^u antigen is tested for in all apparently R' and R'' bloods, by carrying out indirect Coombs tests using three strong incomplete anti-D sera, selected for their ability to react well with D^u cells.

think that perhaps both child and father were $R_0^u r$. A further sample of the husband's blood gave a positive indirect Coombs test with incomplete anti-D sera showing that this was indeed the case.

The husband's blood had not been tested for D^u in the first place, since we do not normally carry out this test when negative results are obtained with anti-C, D, and E sera owing to the low frequency of R_0^u in Caucasian populations.

It is difficult to know what report to give when blocking occurs; one hesitates to report the infant as D positive when the D antigen has not actually been demonstrated, although if the mother has anti-D and the infant has a strongly positive direct Coombs test it is reasonable to suppose that it is D positive. Our usual practice in such cases is to test the infant's cells with anti-C and anti-E, one of which almost always gives a positive result. We then report the infant as "Presumably D positive; D antigen blocked". The C and E antigens are seldom if ever blocked.

Tests with Other Anti-Rh Sera. These tests, often mis-named "genotyping" tests, are carried out on the cells of husbands, of mothers with antibodies and of the infants of mothers with antibodies. In the case of D negative women with antibodies, the tests do not contribute information which is of value in the management of the case. They are done because it is of interest to know of R' and R" women with antibodies, and because of the interesting, but remote, possibility of encountering a woman of Rh type D^u with antibodies in her serum. In the case of D positive women with antibodies these tests are, of course, an integral part of the identification of the antibody, and in the case of antibodies of the other blood group systems, appropriate tests would have to be made according to the blood group system involved. In the case of husbands, these tests are done in order to see whether the husband is likely to be homozygous or heterozygous for the D antigen.

The proportion of those of each Rh phenotype who are homozygous DD can readily be calculated from the genotype frequencies, but such a calculation would only apply to the general population. The fathers of children suffering from haemolytic disease of the newborn are a selected group and have a higher proportion of homozygotes than the general population. The reason for this is that the mothers do not usually produce Rh antibodies until they have had more than one Rh positive pregnancy. This means that those who have antibodies are more likely to have homozygous DD husbands. The exact extent of this effect cannot readily be calculated on theoretical grounds. Our [illegible] 411 fathers [illegible] are given.

[illegible] these tests, a careful watch should always be kept for weak results with the sera and for anything else which appears

of the cross-matching procedure. We determine these groups partly in order to collect information regarding ABO incompatibility and the effect of ABO incompatibility on immunization to Rh and partly because it is convenient to know the ABO groups of the patients with whom one is dealing.

D TYPING. The main thing is to be sure to use a control of the patient's cells, otherwise false positive results due to autoagglutinins, rouleaux, infected and contaminated samples and the like, will inevitably occur sooner or later. A false positive Rh test may be a very serious matter since it may lead to the transfusion of D positive blood with subsequent development of antibodies, or it may lead to no test for antibodies being carried out where these are present. Indeed we have encountered more than one case of failure to predict haemolytic disease of the newborn antenatally due to incorrect D typing of the mother. A saline control would appear adequate here, although a control which substitutes AB serum for the anti-D serum might perhaps offer a slight advantage.

The cells of an infant suffering from haemolytic disease of the new-born due to anti-D are sometimes so coated with incomplete anti-D as to be inagglutinable by saline anti-D. This may cause one to think that the child is Rh negative. A good deal of incomplete antibody is needed to cause the phenomenon, so that it is only seen in certain cases and only when the direct Coombs test is strongly positive. With small amounts of incomplete antibody on the cell surface, partial blocking may occur, and the cells will then be found to give weak results with some saline anti-D sera and negative results with others. Such partially blocked cells resemble D^u cells in their behaviour. If a child has a normal D antigen the blocking is very seldom absolutely complete, if potent agglutinating sera are used. A knowledge of this proved useful in one rather unusual case which we encountered.

CASE 33

An Rh negative (rr) woman had incomplete anti-D in her serum, due, we supposed, to a transfusion of Rh positive blood which she had received previously. Two samples of her husband's blood had been tested and both had given negative results with anti-C, anti-D and anti-E sera, so that it was not anticipated that the child would be affected by haemolytic disease. We were, accordingly, very surprised to find that the infant was jaundiced and anaemic and had a strongly positive direct Coombs test. Its cells gave no agglutination with anti-C, anti-D or anti-E sera, but an eluate of the cells contained anti-D. Illegitimacy was at first suspected, but the absence of any reaction with anti-C or anti-E and the fact that not the faintest trace of agglutination could be seen with the child's cells, even using our most potent saline agglutinating anti-D sera, made us

Homozygous -D-/-D- cells are agglutinated in saline suspension by most incomplete anti-D sera so that a false positive result is usually obtained with anti-C and anti-E agglutinating sera, if these also contain incomplete anti-D. Heterozygous -D- cells show the same effect to a lesser extent. The homozygote -D-/-D- is extremely rare, but Strong D cells, which behave in a similar manner to heterozygous -D- cells, are by no means so rare and are liable to give false positive results in the same way (Table 59). This is shown by the following case.

CASE 34

Mrs. S.W. was D-negative, with anti-D in the serum. Her husband's blood gave the following results:

anti-C	.. ..	v
anti-D	.. ..	v
anti-E	.. ..	++
anti-c	.. ..	+++

These results would indicate that his Rh group was R_1R_2, but the weak result with the anti-E serum gave rise to suspicion as it was known that this serum usually gave much stronger results. His cells were accordingly retested with several other anti-E sera with uniformly negative results. It was then noted that the original anti-E serum was an anti-E + incomplete anti-D serum, and further tests showed that his cells were agglutinable by certain incomplete anti-D sera. It was therefore decided that his Rh group was R_1r (Strong D).

ANTIBODY DETECTION TESTS

The tests used for the detection of antibodies should, so far as possible, be designed to detect as large a range of antibodies as possible and not merely antibodies of the Rh system. We favour the use of two different types of test, partly because antibodies are, themselves, so varied in their behaviour, some reacting better in one test and some in another, and partly because the tests themselves are subject to technical error. The two tests used are the papain cell test and the antiglobulin test.

THE PAPAIN CELL TEST (see Chapter II). The cells used in the test are a mixture of equal parts of R_1 cells and R_2 cells. These may be R_1r and R_2r or, somewhat preferably, R_1R_1 and R_2R_2. This mixture is more sensitive in the detection of anti-C, E, c and e than is the use of R_1R_2 cells alone.

The cells selected should also bear as many of the other blood group antigens as can be managed, paying particular attention to those whose antibodies react with papainized cells. In any case, one of the cells should be P positive, and one should be Le(a+b−) and the other Le(a−b+). If suitable individuals who are willing to be bled at

unusual, and all such matters should be subjected to further investigation. The occasional occurrence of Strong D, or -D- in husbands, for example, can be misleading at times.

TABLE 58. RH GROUPS OF FATHERS OF CHILDREN SUFFERING FROM HAEMOLYTIC DISEASE DUE TO ANTI-D
(Showing that a high proportion are homozygous)

Group		*Number observed*		*Number expected in 1,411 random D positive individuals*	
Probably homozygous	R_1R_1	423		314	
	R_1R_2	338		231	
	R_2R_2	8		6	
		Total	769	Total	551
Probably heterozygous	R_0	18		35	
	R_1r	439		592	
	R_2r	31		33	
		Total	455	Total	660
	*R_2	184		200	
		Total	184	Total	200
Grand totals ..			1,411		1,411

* Not tested with anti-e.

TABLE 59. FALSE POSITIVE AGGLUTINATION USING STRONG D CELLS WITH ANTI-C AND ANTI-E SERA CONTAINING INCOMPLETE ANTI-D
(Renton and Hancock, 1956)

Red cells	*Anti-C*		*Anti-E*	
	Na	*549*	*375*	*341*
Strong D:				
Sw. R_2r ..			+	++
Ha R_2r ..	++	+	—	++
Hn R_2r ..			—	+
Normal D:				
R_1R_1 ..	v	v	—	—
R_1r ..	v	v	—	—
R_2R_2 ..	—	—	+++	v
R_2r ..	—	—	+++	v

Homozygous -D-/-D- cells are agglutinated in saline suspension by most incomplete anti-D sera so that a false positive result is usually obtained with anti-C and anti-E agglutinating sera, if these also contain incomplete anti-D. Heterozygous -D- cells show the same effect to a lesser extent. The homozygote -D-/-D- is extremely rare, but Strong D cells, which behave in a similar manner to heterozygous -D- cells, are by no means so rare and are liable to give false positive results in the same way (Table 59). This is shown by the following case.

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anti-C	..	..	v
anti-D	..	..	v
anti-E	..	..	++
anti-c	..	..	+++

These results would indicate that his Rh group was R_1R_2, but the weak result with the anti-E serum gave rise to suspicion as it was known that this serum usually gave much stronger results. His cells were accordingly retested with several other anti-E sera with uniformly negative results. It was then noted that the original anti-E serum was an anti-E + incomplete anti-D serum, and further tests showed that his cells were agglutinable by certain incomplete anti-D sera. It was therefore decided that his Rh group was R_1r (Strong D).

ANTIBODY DETECTION TESTS

The tests used for the detection of antibodies should, so far as possible, be designed to detect as large a range of antibodies as possible and not merely antibodies of the Rh system. We favour the use of two different types of test, partly because antibodies are, themselves, so varied in their behaviour, some reacting better in one test and some in another, and partly because the tests themselves are subject to technical error. The two tests used are the papain cell test and the antiglobulin test.

THE PAPAIN CELL TEST (see Chapter II). The cells used in the test are a mixture of equal parts of R_1 cells and R_2 cells. These may be R_1r and R_2r or, somewhat preferably, R_1R_1 and R_2R_2. This mixture is more sensitive in the detection of anti-C, E, c and e than is the use of R_1R_2 cells alone.

The cells selected should also bear as many of the other blood group antigens as can be managed, paying particular attention to those whose antibodies react with papainized cells. In any case, one of the cells should be P positive, and one should be Le(a+b−) and the other Le(a−b+). If suitable individuals who are willing to be bled at

frequent intervals cannot be found, it is not too difficult to meet these criteria by screening tests on random bloods at say weekly intervals. The test detects anti-Rh, anti-P, anti-Le^a, anti-Le^b, anti-H, anti-O and occasional autoagglutinins. If repeated in capillaries at 37° C., it is found that the vast majority of positive results are due to Rh antibodies.

In most cases, where the papain test is positive, the Coombs test will be found positive also, so that antibody identification tests can proceed normally. Occasionally, however, the papain slide test is positive at room temperature but the Coombs test is negative. In such cases the identification of the antibody should be carried out by the usual method but tests against papainized standard cells must, of course, be included.

Whilst Rh antibodies which are only detectable by the papain test are often so weak as to be of little clinical significance, it ought not to be assumed that this is invariably the case, since a weak antibody will sometimes become much stronger, even during the last few weeks of pregnancy. Furthermore, antibodies giving unusual patterns of reaction in the test are occasionally encountered and the following case is an example of this:

CASE 35

Mrs. D.C. group A, rr.
Husband, group A, R_1r.
1st child: born 9.12.47, normal.
2nd child: born 29.1.52, anaemic but recovered; grp. A, R_1r.
Direct Coombs test ++++
3rd child: born 27.11.57; jaundiced and anaemic at birth.
Cord haemoglobin, 11·5 gm.%; serum bilirubin, 4·4 mgm.%.
Group A, R_1r. Direct Coombs test +++++
Exchange transfusion, but died 28.11.57. Postmortem showed atelectasis.

The maternal serum contained anti-D, which reacted as follows:

Date	*Papain*	*Coombs test*	*Serum albumin 37° C.*	*Saline 37° C.*	*Saline 16° C.*
3.10.51 ..		–			
4.12.51 ..		++	+++	–	–
31.1.52 ..	++++	+	++	w	–
22.1.53 ..	+++++	++	–	–	–
5.6.56 ..	+++++	–	–	–	
15.7.57 ..	++++	–	–	–	–
29.8.57 ..	++++	w	+	–	–
24.10.57 ..	++++	+	+	–	–
26.11.57 ..	+++++	+++++	+	–	–

Most of these tests were carried out using at least R_1, R_2 and three or more rr bloods. On several occasions a series of ten bloods of various Rh groups was used and the Coombs tests carried out using as many as ten different batches of Coombs reagent (including an anti-non-γ-globulin reagent). These measures confirmed the accuracy and specificity of the results.

Without the use of the papain test it would have been very difficult to know what was happening, since the positive results which were obtained in the Coombs and serum albumin tests, prior to the birth of the second child, and subsequently when not pregnant, disappeared at a later date. These tests were only doubtfully positive a month before the birth of the third child. We certainly did not expect a strongly positive indirect Coombs test one day before delivery, nor a severely affected child.

THE INDIRECT COOMBS TEST. This test, like the papain test, ought to be done using cells containing as many antigens as can be managed. However, it is not desirable to use a mixture of cells from a large number of different blood samples, owing to the danger of overlooking agglutinations which only involve a small proportion of the cells. A mixture of not more than two, or at the most three, bloods should be used. R_1 and R_2 cells should be included and one of the cells should also be Kell-positive, and it is also desirable to include the antigens M and S if possible. It is, perhaps, an advantage not to use the same two bloods as are used in the papain test.

We have never encountered an Rh antibody giving a positive result in the antiglobulin test and a negative one in the papain test.

ANTIBODY IDENTIFICATION TESTS

The identification of antibodies, once they have been detected, is dealt with more fully elsewhere (see Chapter XII), so that there are only a few points which need to be touched on here. The first of these is the choice of cells to be used in the test. In identifying an unknown antibody, the methods used have to be adapted to the particular circumstances of the case. We find, nevertheless, that the procedure enumerated above, namely Coombs test, serum-albumin test and saline agglutination tests at 16° and 37° C. with R_1, R_2, R', R", and three or more selected rr bloods, is extremely useful as an initial routine procedure.

If the antibody is anti-D, anti-C+D, or anti-D+E, these tests may themselves be sufficient to identify the antibody (p. 272) and give also a very good idea of its strength and mode of reaction. If antibodies of other specificities are present, the tests give a good indication of the mode of reaction and strength of the antibody and often a useful guide as to its specificity. It is usually easy to see from the results of these

tests what further tests will be needed to carry the identification of the antibody to its conclusion.

TESTS ON INFANTS

THE DIRECT COOMBS TEST. Two important points regarding this test are, firstly, to use a saline control for the detection of auto-agglutinins, Wharton's Jelly, etc., and secondly, not to fall into the error of attempting to wash such a large volume of cells that adequate removal of the serum is not obtained.

In ABO haemolytic disease, the direct Coombs test is frequently negative or only weakly positive; if the latter, it does not usually remain so for more than a few days. The results of the direct Coombs test should always be considered in relation to the Rh type of the infant and to the presence or absence of antibodies in the maternal serum. The use of Coombs reagents of the anti-non-γ-globulin type will often help to clear up discrepancies as is illustrated by the following case:

CASE 36

Mrs. F. was found D-positive during the antenatal period: her serum was not tested for antibodies. She was delivered of a child whose cord blood had a positive direct Coombs test. A further sample of blood was accordingly requested from the mother, which was confirmed as D-positive (R_2). No antibodies could be detected in her serum, in spite of extensive tests, but it was noted that the Berger Kahn test was strongly positive. Further tests on the infant's cells with different Coombs reagents showed that they were strongly agglutinated by anti-non-γ-globulin reagents, but not by anti-γ-globulin reagents. The reactions were probably associated with maternal syphilis.

CASE 37

Mrs. A.M. was also D-positive, R_1R_1, and her Berger Kahn test was negative. The infant's cells gave the same reactions with anti-globulin reagents as in the preceding case. Tests on a post-partum sample of the mother's blood failed to reveal the presence of any specific antibody other than a strong cold incomplete anti-H. The child was clinically and haematologically normal and remained so.

OTHER TESTS. The grouping tests of the infants' erythrocytes have been dealt with already, but there are a few other tests we occasionally use in special cases. In ABO haemolytic disease the cells of the infant will sometimes agglutinate when suspended in fresh adult serum (Witebsky *et al.*, 1947) and anti-A or anti-B can sometimes be demonstrated in the infant's serum by the papain or antiglobulin techniques. Occasionally it is useful to prepare an eluate of the infant's cells and to examine the specificity of the eluted antibody. All these methods are described in other chapters.

Investigation of Cases with Suspicious Histories

Cases with a history suggestive of the presence of an antibody or of the occurrence of haemolytic disease in the past need further investigation. It is not likely to be practicable to carry out all possible tests in all such cases, and in consequence a compromise has to be adopted; we divide them into two classes, according to the degree of suspicion to which the history gives rise. The first class comprises the less suspicious cases, and the second the more suspicious ones. The division of the cases into these two classes is arbitrary, since the extent to which the tests can be carried out is dependent on the financial and technical resources of the laboratory concerned. The division is also dependent on the amount of information available about the cases. The division which we ourselves adopt, and the tests which we carry out, are as follows:

The Less Suspicious Cases

These comprise women giving a history of blood transfusion not followed by haemolytic reaction or a history of intramuscular injection of blood; also cases where the information received is scanty, and suggests, but not strongly, the possibility of haemolytic disease. Families with mentally defective children, or children who died from unknown causes in the neonatal period, are included in this classification.

In all such cases the mother's serum is tested

(*a*) by the papain test using the standard mixture of cells described above; and,

(*b*) by the Coombs test using R_1, R_2, and three or more selected rr bloods carrying as many of the blood group antigens as can be managed.

The More Suspicious Cases

These comprise all cases where there is a reasonable suspicion that haemolytic disease might have occurred in the past. Women who have had stillborn, jaundiced or anaemic infants or oedematous infants are included in this category, as are post-partum cases where the condition of the child is clinically or haematologically suggestive of haemolytic disease of the newborn. If the mother gives a history of blood transfusion, or intramuscular injection of blood followed by an unsuccessful pregnancy or a jaundiced or anaemic child, she should certainly be included in this category.

In such cases [illegible]

[illegible] from the mother. A fresh sample of blood

tests what further tests will be needed to carry the identification of the antibody to its conclusion.

TESTS ON INFANTS

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child's cells are of an ABO group compatible with the mother's serum and the father's cells are incompatible. In such cases the mother's serum could only be tested against the father's cells after neutralization with A or B substances, and the difficulties of the procedure outweigh the risks arising from under-development of antigen on the child's cells.

When the cells of both father and child are incompatible with the mother's serum on the ABO system, joint testing is more difficult, and the question of ABO haemolytic disease also arises. Tests of the maternal serum against a series of standard cells covering as many of the known blood group antigens as can be managed, combined with tests for immune anti-A and anti-B in the mother's serum, are usually more convenient than joint tests against the cells of the father or child, though it should not be forgotten that such tests would fail to reveal haemolytic disease due to a "family" antigen, or to a rare antigen such as C^x. It is, therefore, desirable at least to include a Coombs test against the husband's cells using the mother's serum with the iso-agglutinin neutralized by secretor saliva, although this is, of course, a test for immune anti-A and anti-B as well as for a "family" antigen. If this test is positive and the test using neutralized serum and A or B cells is negative, this would suggest a "family" antigen. This deduction is not conclusive, however, as the following cases illustrate:

CASE 38

Mrs. A.N. was group OR_1r, and her husband was group A_1R_1r. No antibodies could be detected in her serum in tests against a [illegible] wife's serum with any group A cells removed the reaction against the husband's cells and it was therefore concluded that the reaction with the husband's cells was due to incomplete anti-A and not to a "family" antibody. The fact that the wife's neutralized serum gave a positive Coombs test with the husband's cells and not with other A cells was misleading in this case, and we thought that it indicated that the husband had an exceptionally strong A antigen.

CASE 39

S.H., a former member of our staff, had exceptionally strongly reacting group A red cells. Whenever a number of group A bloods were tested with anti-A_1 it was always found that the cells of S.H. were agglutinated a good deal more strongly than any of the others.

A Possible Alternative System of Antenatal Testing

The antenatal testing carried out in our laboratory is based on the premise that one should first find out which women are D negative,

from the mother should be sent at the same time so that the serum can be tested against the father's or child's cells whilst still fresh.

A full-scale investigation should then be carried out as follows:

(1) Mother, father and/or child; ABO group, anti-A_1 (if appropriate), two anti-D sera, anti-C, anti-E, anti-c, anti-e (if appropriate and available), saline control.

Test for D^u if positive with anti-C, or anti-E and negative with anti-D.

(2) If ABO group of mother is compatible with that of father, or child, carry out joint tests between the mother's serum and the cells of the father or child by papain, serum albumin, Coombs, and by saline agglutinations at 16° C. and 37° C.

(3) If ABO group of mother is incompatible with father and child, test the mother's serum by the following methods:

(*a*) Papain test, using the standard mixture of cells.

(*b*) Coombs, serum albumin, saline agglutination tests at 16° C. and 37° C. with R_1, R_2 and three or more selected rr bloods, carrying as many of the known blood group antigens as can be managed.

(*c*) Tests for immune anti-A or anti-B, or both as applicable, as detailed on p. 133.

(*d*) Neutralize the mother's anti-A or anti-B agglutinins with secretor saliva and test against the cells of the father or child. A control using A (or B, if appropriate) cells is essential. This control, of course, is the same as one of the tests for immune anti-A.

By the time all the tests described above have been carried out, it will generally be found that enthusiasm has waned to the extent that tests of the neutralized maternal serum against the father's cells will only be carried out by the Coombs technique. They can also be done by the serum albumin and saline agglutination techniques if desired; the papain technique usually proves impracticable owing to the difficulty of neutralizing the anti-A and anti-B agglutinins to such an extent that they will not react with A and B papainized cells.

Haemolytic disease of the newborn is due to an antibody in the mother's serum reacting with an antigen on the child's cells which it has inherited from the father, so that if the ABO groups are compatible and the joint tests are negative, haemolytic disease of the newborn is virtually excluded. In carrying out joint tests it is, generally speaking, better to use the cells of the father rather than those of the child, owing to the fact that some blood group antigens are not fully developed at birth; the reactions are more readily detected using adult cells. On occasion, however, it is preferable to use the child's cells, either because there is some doubt about the paternity of the child, or because the

child's cells are of an ABO group compatible with the mother's serum and the father's cells are incompatible. In such cases the mother's serum could only be tested against the father's cells after neutralization with A or B substances, and the difficulties of the procedure outweigh the risks arising from under-development of antigen on the child's cells.

When the cells of both father and child are incompatible with the mother's serum on the ABO system, joint testing is more difficult, and the question of ABO haemolytic disease also arises. Tests of the maternal serum against a series of standard cells covering as many of the known blood group antigens as can be managed, combined with tests for immune anti-A and anti-B in the mother's serum, are usually more convenient than joint tests against the cells of the father or child, though it should not be forgotten that such tests would fail to reveal haemolytic disease due to a "family" antigen, or to a rare antigen such as C^x. It is, therefore, desirable at least to include a Coombs test against the husband's cells using the mother's serum with the iso-agglutinin neutralized by secretor saliva, although this is, of course, a test for immune anti-A and anti-B as well as for a "family" antigen. If this test is positive and the test using neutralized serum and A or B cells is negative, this would suggest a "family" antigen. This deduction is not conclusive, however, as the following cases illustrate:

Case 38

Mrs. A.N. was group OR [illegible] her husband was group A_1R_1r. [illegible] n her serum in tests against a [illegible] when her serum was [illegible] with secretor saliva it was fo[illegible] test with the husband's cells, [illegible]
It was found that absorption of the wife's serum with any group A cells removed the reaction against the husband's cells and it was therefore concluded that the reaction with the husband's cells was due to incomplete anti-A and not to a "family" antibody. The fact that the wife's neutralized serum gave a positive Coombs test with the husband's cells and not with other A cells was misleading in this case, and we thought that it indicated that the husband had an exceptionally strong A antigen.

Case 39

S.H., a former member of our staff, had exceptionally strongly reacting group A red cells. Whenever a number of group A bloods were tested with anti-A_1 it was always found that the cells of S.H. were agglutinated a good deal more strongly than any of the others.

A Possible Alternative System of Antenatal Testing

The antenatal testing carried out in our laboratory is based on the premise that one should first find out which women are D negative,

from the mother should be sent at the same time so that the serum can be tested against the father's or child's cells whilst still fresh.

A full-scale investigation should then be carried out as follows:

(1) Mother, father and/or child; ABO group, anti-A_1 (if appropriate), two anti-D sera, anti-C, anti-E, anti-c, anti-e (if appropriate and available), saline control.

Test for D^u if positive with anti-C, or anti-E and negative with anti-D.

(2) If ABO group of mother is compatible with that of father, or child, carry out joint tests between the mother's serum and the cells of the father or child by papain, serum albumin, Coombs, and by saline agglutinations at 16° C. and 37° C.

(3) If ABO group of mother is incompatible with father and child, test the mother's serum by the following methods:

(a) Papain test, using the standard mixture of cells.

(b) Coombs, serum albumin, saline agglutination tests at 16° C. and 37° C. with R_1, R_2 and three or more selected rr bloods, carrying as many of the known blood group antigens as can be managed.

(c) Tests for immune anti-A or anti-B, or both as applicable, as detailed on p. 133.

(d) Neutralize the mother's anti-A or anti-B agglutinins with secretor saliva and test against the cells of the father or child. A control using A (or B, if appropriate) cells is essential. This control, of course, is the same as one of the tests for immune anti-A.

By the time all the tests described above have been carried out, it will generally be found that enthusiasm has waned to the extent that tests of the neutralized maternal serum against the father's cells will only be carried out by the Coombs technique. They can also be done by the serum albumin and saline agglutination techniques if desired; the papain technique usually proves impracticable owing to the difficulty of neutralizing the anti-A and anti-B agglutinins to such an extent that they will not react with A and B papainized cells.

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Reference

Witebsky, E., Rubin, M. I., Engasser, M., and Blum, L. (1947). Studies in erythroblastosis foetalis. II. Investigation on detection of sensitization of red blood cells of newborn infants with erythroblastosis foetalis. *J. Lab. and Clin. Med.*, 32, 1339.

and then test their sera at intervals for the development of antibodies. The D positive women are examined to a lesser extent, depending on their history. The reasons for the prevalence of this system are partly historical and depend on the fact that anti-D is not only the commonest cause of haemolytic disease, but also the first to be discovered. The system also derives from the fact that in the past it has been easier to test the cells for the D antigen than to test the serum for anti-D. The papain slide test, however, offers a very convenient and simple method of detecting Rh antibodies of all types, and it will detect many other antibodies also, so that it could be argued that the time devoted to the large-scale D typing of antenatal patients would be better devoted to testing their sera with papain cells. This would also reduce the demand for anti-D serum, a demand which it is always difficult to meet. Under this system each antenatal patient would have a blood test consisting of: haemoglobin level, syphilis test, and papain cell test. These tests could be carried out when pregnancy was first diagnosed and repeated about four weeks before term, and the blood of those patients who gave positive results in the tests would be subject to further investigation. In the case of those bloods giving positive results in the papain cell test, the investigation would include identification of the antibody and D typing of the mother. It could, of course, be argued that the large-scale D typing of antenatal patients is carried out not only in order to detect cases of haemolytic disease but also so that the information is available in case the mother requires transfusion when delivered, but we would suggest that the D typing of the mothers for this purpose could equally well be carried out as part of the cross-matching test, or at least it could be carried out on admission to hospital. There does not now appear to be any good reason for D typing all antenatal patients, many of whom will never require a transfusion, any more than it is necessary to D type all persons suffering from peptic ulcer in case they have a haematemesis. Cases with histories suggestive of haemolytic disease of the newborn would still require intensive investigation as at present, but these are likely to be D positive as well as D negative and could be referred for the special investigation on clinical grounds. The proposed system would, we suggest, effect a very considerable saving in every respect, and would detect anti-D at least as well as the present system, and a good many antibodies of other specificities as well. Furthermore under the present system, if a D negative woman is falsely grouped as D positive it is likely that her serum will never be tested for antibodies in this or subsequent pregnancies. We have seen a number of cases of this, where a child severely affected with haemolytic disease was born at home instead of in hospital in consequence and where the cause of its condition was not diagnosed for some days. If the serum of all antenatal patients was tested twice during each pregnancy as suggested these unfortunate missed cases would be less likely.

Blood transfusions may be given to a patient which are apparently compatible by the in vitro test described, but which do not survive normally. This may be for unknown reasons, or because the antibody has not been detected, or because it is not present, owing to the fact that transfused cells containing the homologous antigen are circulating in the patient's blood.

It should be remembered that the cross-matching test does not prevent immunization of the patient. This is very likely to occur if, for example, Rh negative recipients are transfused with Rh positive blood, and consequently, special steps are taken to avoid this by ensuring that persons who are Rh negative receive Rh negative blood whenever possible.

Summarizing, therefore, from a practical point of view the cross-matching test is intended to:

1. Detect the presence of atypical antibodies in the patient's serum, active against red cells which it is proposed to transfuse. These atypical antibodies might have arisen as the result of immunization due to previous blood transfusions, intramuscular injections of blood or pregnancy.
2. Detect errors in ABO grouping of the donor and recipient.

The cross-matching test will not:

1. Prevent immunization of the patient.
2. Detect errors of Rh typing of donor and recipient (unless the recipient's serum contains Rh antibodies).

Samples

PATIENT

Samples of blood from the patient should be in the form of clotted blood. 20 ml. of clotted blood should be collected aseptically and placed in a sterile container labelled so as to be able exactly to identify the patient. The red cells should be separated from the serum, washed once in saline (or better, twice) and grouped for ABO and Rh as described in Chapters VI and VIII. If time permits, samples may be stored at 4° C. overnight, and this will diminish difficulties due to auto-agglutinins.

The patient's [illegible] th[illegible] inf[illegible] and, secondly, so that any haemolysis occurring in the cross-matching test will be observed.

Serum should be sterile and if it is to be preserved for a few days it should be preserved frozen solid. A sample of blood from a patient should be of sufficient volume to cover the first series of transfusions.

CHAPTER X

THE CROSS-MATCHING TEST

THE cross-matching (or compatibility) test is performed immediately prior to blood transfusion and the donor's cells are tested directly against the recipient's serum to ensure that the blood which is to be transfused is suitable for the patient. In its entirety, it comprises the ABO and Rh grouping of the donor and patient, together with the direct matching test. This chapter, however, is concerned only with the latter.

It is normal practice to select for cross-matching, blood of homologous ABO and Rh group, subject to certain provisos. That is to say, if a patient is group A Rh negative, normally he or she would be transfused with group A Rh negative blood. Under certain circumstances, however, if there is a shortage of Rh negative blood, male patients may be transfused during the first series of transfusions with Rh positive blood, and, furthermore, under certain emergency circumstances group O blood may have to be used. For therapeutic purposes, especially in haemolytic disease of the newborn either due to Rh antibodies or to ABO incompatibility, blood of a different group from that of the patient may be transfused.

The cross-matching test, as normally performed, shows that no antibodies in the patient's serum are active against the red cells which it is proposed to transfuse. The object of this is to ensure that, in vivo, the transfused cells will survive normally. The test, therefore, safeguards patients against abnormally rapid destruction of the transfused cells which may be so rapid as to cause a frank haemolytic reaction, or may proceed slowly so that a poor therapeutic result is obtained, or may be anywhere between these two extremes, both of which are, obviously, undesirable. It is important, therefore, when considering the cross-matching test, to bear in mind the work that has been done, particularly by Mollison (1951) on the in vivo survival of incompatible red cells in patients whose sera contain antibodies of various kinds. This experience enables one to know which antibodies are likely to be dangerous and whether some are more dangerous than others. It is possible to relate in vivo survival to the results obtained by in vitro tests on these antibodies.

After each blood transfusion, careful watch should be kept for the failure of the transfused blood to survive normally. This may be done by haemoglobin tests or, better still, by examination of the serum twelve hours after transfusion for the appearance of methaemalbumin. Any sign of decreased red cell survival should be followed by careful examination of the patient's serum.

CROSSMATCHING INVESTIGATION

Serial No. L 2109

PATIENT'S SURNAME WELLER CHRISTIAN NAMES Martha DATE OF BIRTH 5 10.26 SEX F

ADDRESS 10, GRANBY ST, MINETOWN

HOSPITAL MINESHIRE ROYAL INFIRMARY

REF No.

DATE SPECIMEN RECEIVED 18 11 57

MEDICAL OFFICER

Reason for Transfusion POST PARTUM ANAEMIA DELIVERED 15 11 57

Hb %	R. B. C.	DATE	Bottles of Whole Blood		Requested
52%		17 11 57	Bottles of Conc. Red Cells	1	

Previous Transfusion and Reactions (if any) 2 PINTS AFTER MISCARRIAGE 1954

GP O Rh +ve AE 627

689

Obstetrical History 1 FULL TERM LIVE BIRTH 1953

1 MISCARRIAGE (12 WEEKS) 1954

1 FULL TERM LIVE BIRTH 1957

Other Remarks BLOOD TESTED DURING ANTE NATAL PERIOD 1957

YOUR REF 729586

FIG 5. Cross-matching card, obverse

For a subsequent series of transfusions, a fresh sample of blood should be obtained on each occasion and the ABO and Rh groups confirmed. A "first series of transfusions" is defined as those transfusions which may be given on any three consecutive days following collection of the sample. This does not necessarily mean that a sample must be taken every third day, and if transfusions are being given at longer intervals as long as possible should be left after one transfusion before the sample is taken for cross-matching any succeeding transfusion. This is in order to give antibodies which may have resulted from immunization by the initial transfusion time to develop. The proviso of three days is put in because the shortest time in which we have seen an anti-Rh antibody develop in the blood of a patient following transfusion is seven days from the first transfusion of blood. In Worssam's (1957) case a woman in whose serum Rh antibodies could not be detected was given 5 ml. of R_1R_1 blood intravenously and anti-D with a titre of 64 was found a week later.

DONOR

Donor blood samples are obtained either by aseptic sampling from the bottle of blood, or from a pilot tube. It must be remembered that in old blood there is some deterioration in the antigenic strength of the red cells. A. S. Wiener pointed out many years ago that it was possible to increase the agglutinability of stored red cells by washing them three times in normal saline. Although normally with whole stored citrated blood removal of plasma followed by washing twice in saline is a satisfactory preparation for the red cells, if the cells are old washing three times is an advantage.

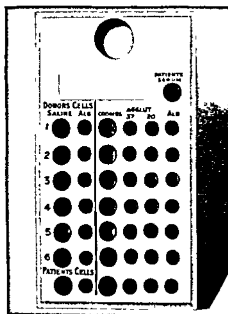
Procedure and Technique of the Cross-matching Test

The technique of a test should be distinguished from the procedure by which it is carried out. The technique consists of the actual serological method which is used, e.g. Coombs Test, Saline Agglutination Test by the tube technique; while the procedure comprises all those steps which are taken to ensure that the technique is carried out correctly, using the correct samples of blood from donor and patient; that the results are correctly recorded and that the correct bottle of blood is despatched to the patient, and so forth. A good procedure is particularly vital in the cross-matching test, since incompatible transfusions arise much more often from failures of the procedure than from failures of the technique.

PROCEDURE

It is important in every Pathological Laboratory, where a number of tests have to be done simultaneously, to make sure that a set procedure is followed so that the risk of confusion and of clerical and other errors

PLATE XV.



a. A cross-matching block. See p. 211. $\times \frac{1}{3}$



b Incompatible transfusion due to anti-c. Direct Coombs test. Only the transfused cells agglutinate. See p. 229. $\times 220$

LABORATORY INVESTIGATION Patient's Name WELLER

DONOR'S CELLS		Stated Group	SIDE TUBE		BOTTLE							PATIENT'S SERUM			
Khan No.	Date Bled		Anti—A	Anti—B	Anti—A	Anti—B	Anti—D	Anti—D				Coombs 245	Agglutination 37	16	Albumin
BK 157	17·11 57	O+	—	—	—	—						—	—	—	—
BK 243	17 11 57	O+	—	—	—	—						—	—	—	—
							MAR	VAL							
PATIENT'S CELLS			—	—			V	V				—	—	—	—
											A			V	
											A				
											B			V	
											O				
											Amount Sent	500 ml conc. cells.			
											Date	19 11·57			

Technician's Signature SRS.

FIG 6. Cross-matching card, reverse.

is minimal. Experience of transfusion reactions has shown that procedural recording and clerical errors are a much greater danger than technical ones (see Chapter V).

Sometimes, of course, procedural recording and clerical and technical difficulties are combined. For example:

CASE 40

A patient was admitted to hospital for hysterectomy and on admission was found to be group O Rh positive. During the transcribing of her group to her record card she was wrongly entered as group A Rh positive. After the operation a bottle of blood was called for and a cross-matching test was performed with a bottle of group A Rh positive blood and no incompatibility was detected. The patient received the group A Rh positive blood and had a severe haemolytic reaction. Subsequently, the cross-matching test was re-done on a pre-transfusion sample of serum and showed an incompatibility. Here the cross-matching test was badly performed and failed to show the incompatibility, but had the previous record been correctly entered the error would have been avoided.

The following recommendations should be followed:

1. The bottle of blood should be sampled and a record made on the sample of the serial number of the bottle so that it can be readily identified. If a pilot tube is used the pilot tube should not be removed from the bottle of blood during this sampling; a small quantity should be aseptically withdrawn into a suitable tube, also labelled with the serial number.
2. The sample of blood from the patient should also be suitably labelled in sufficient detail to identify the patient correctly.
3. A special cross-matching rack should be used (Plate XVa).
4. Records of the cross-matching test should be kept on a special card (Figs. 5 and 6), or in a cross-matching register.
5. During the reading of the cross-matching test the results should be written on the card (or cross-matching register) as the tests are read, and if the test shows compatibility a suitable compatibility label, with sufficient detail to identify the patient for whom the bottle is intended should be stuck on to the bottle of blood. These details should be similar to those on the sample from the patient.
6. Keep the cross-matched blood in a separate place from the main stock of blood.

Plate XVa shows a cross-matching block which is recommended for use. Such a block may be arranged to suit the local needs of the laboratory. It will be seen that there are places for the sample of the patient's blood, for tubes containing specimens of the donor's cells and recipient's cells and serum, and (on this particular block) a small

in the refrigerator. During the night, however, an inexperienced nurse came down to the refrigerator which was used as the Blood Bank, opened it and removed a bottle of blood thinking it was the one that had been prepared for the patient, and this was given, resulting in a haemolytic reaction.

Two lessons are learned from this: (1) that bottles should never be given without compatibility labels being attached, and (2) that had it been known that a special section of the refrigerator was kept for cross-matched blood, as distinct from the ordinary Blood Bank, the difficulty might have been avoided.

A card index system for recording cross-matching tests is preferable to a cross-matching register, for the following reasons:

1. More information can be put on a cross-matching card than in the register. The obverse side of the card contains details of the patient's past history and past transfusion history; the reverse side of the card contains full technical details, the reaction of each tube being shown.

2. With each subsequent series of transfusions a fresh card is prepared by the Records Office and submitted to the technician with the appropriate blood sample from the patient, together with the previous cards for that particular patient. This enables the complete transfusion history of that patient to be quickly obtained for the doctor's or technician's benefit.

3. In a card index system the complete records concerning a particular patient are more readily obtained, if difficulties arise in the future, than if one has to scan through the pages of a large book.

4. They are more readily handled and dealt with in the laboratory, each card being placed either under or nearby the cross-matching block and dealt with individually on each occasion on which the cross-matching test is read by the technician.

Technique

Briefly it may be said that the cross-matching test consists of testing the patient's serum against the cells of each donor by various methods, including control tests of the patient's serum against his own cells by the same methods. These controls are essential for the elucidation of various difficulties which occur in the cross-matching test. It is recommended that the test should be carried out using the following methods:

1. Antiglobulin test at 37° C.
2. Tube saline agglutination test at room temperature or in a 16° C. incubator.
3. Tube saline agglutination test at 37° C.

The antiglobulin test should be carried out according to the method described in Chapter IV, using a period of 35–45 minutes incubation.

window at the top of the block on which the patient's name can be written. Sufficient holes are available for six lots of donor blood to be included. One block is intended for the cross-matching of one order of blood for one patient. Places are available on this particular rack not only for saline agglutination and antiglobulin tests but also for the serum albumin test, because most of the cross-matching tests done in the Blood Transfusion Laboratory are on difficult cases where the test is sometimes useful. However, these could be omitted.

When the tests have been set up, those tubes which require incubation at 37° C. are removed from the block and placed in the corresponding hole of an exactly similar block labelled with the patient's name, this block having been previously warmed in the 37° C. incubator. The two blocks, with their tests, are now incubated at the appropriate temperatures following which the results are read. The block which is used at 37° C. may with advantage be made of metal, so that the 37° C. tests can be carried out in the water bath. The system of interlocking blocks, fastened together by pins, similar to that recommended for large-scale ABO grouping, can be used if desired, but if this is done one has to be careful not to make the mistake of pinning all the blocks together after the period of incubation and then reading all the results, since this would be liable to lead to results being read after the tubes had reached room temperature instead of at the temperature intended. Tests should always be read as nearly as possible at the temperature at which they have been incubated.

The main purpose of the blocks is to have together in one place all specimens concerning the particular cross-matching for that patient.

Whilst bottles of blood are being cross-matched they should be segregated in a special refrigerator, or a special part of the refrigerator, until the cross-matching test is completed. During the reading of the test the results are filled in on the cross-matching card or register and finally completed and signed by the doctor or technician responsible. It is his or her duty then to fill in and afix the compatibility labels to the bottles and put them in that section of the refrigerator reserved for cross-matched blood. Cross-matched blood should always be segregated from blood which is not so labelled. This is especially important in hospitals because people may inadvertently remove from a refrigerator a bottle of blood which has not been cross-matched, and if it is known that cross-matched blood is segregated and put in a special place, this difficulty is avoided.

CASE 41

Mrs. A. required blood transfusion. She was group B. Rh positive. It had been known that she was likely to require blood transfusion [illegible]

cross-matching block. Avoid clots as in (6) below if time permits.

4. Determine the patient's ABO and Rh groups by the techniques described in Chapters VI and VIII.
5. Obtain the requisite number of bottles of donors' blood of the same ABO and Rh groups as the patient, and suspend the cells in saline in 3 in.$\times\frac{1}{2}$ in. tubes, placing the tubes in the appropriate holes of the block.
6. Let the donor's cell suspension stand for a few minutes to allow the clots to settle at the bottom of the tubes. Remove the top portion into another tube, wash twice and prepare a 10 per cent suspension.
7. Set up the antiglobulin tests.
8. Dilute the cell suspension to 3 per cent and set up the agglutination tests.
9. Incubate the tests at the appropriate temperatures.
10. Wash the cells for the antiglobulin tests, and carry out the tests.
11. Read the agglutination tests, centrifuging if required. If all the tests are negative, the cross-matching card should be signed by the technician concerned, and the bottles of blood labelled and issued.
12. If some of the tests are positive further investigations will be called for which are discussed in a subsequent section of this chapter.

Using this method and employing the centrifugation technique, compatible blood can be provided in about one hour. If it is essential to provide blood more rapidly than this, emergency techniques, discussed at the end of this chapter, will have to be employed.

Value and Purpose of the Cross-matching Test

In this section we shall consider whether the test is necessary at all; then its purpose, and finally the means by which the value of a particular method can be assessed.

Necessity for the Cross-matching Test

If absolute reliance could be placed on the ABO and Rh typing of the donor and the recipient so that they were always correct, and homologous blood was selected for transfusion, would it, under such circumstance, be possible to omit the cross-matching test? It would not be so, since there might still be in the serum of the patient atypical antibodies which might give rise to incompatibility and which it is important to detect. It might be argued, however, that the cross-matching test, under these circumstances, could be restricted to persons who had received previous parenteral injections of blood and to females who

A centrifuge saline agglutination technique is used after a period of 40 minutes incubation. Alternatively, a sedimentation method in tubes allowing 1½–2 hours before the results are read, may be employed, together with a longer incubation time in the antiglobulin test.

A saline agglutination technique employing a centrifugation of 500–1,000 r.p.m. for 2–3 minutes is suitable. The advantages of centrifugation are, firstly, that it speeds up the test and, secondly, that it increases the strength of agglutinations of all types. Indeed, the agglutination due to certain antibodies, anti-Lewis in particular, is markedly improved by centrifugation. The disadvantages are, firstly, that it is an additional step, secondly, it introduces a danger of accidentally getting the tubes in the wrong order, and thirdly, if centrifugation is excessive, it can make the test a little difficult to read.

It is recommended that normally the saline agglutination centrifuge technique be used with the indirect antiglobulin test of 35–45 minutes incubation. Where time is not important and large numbers of cross-matching tests are being done, the sedimentation method may be used, and under such circumstances the time of incubation in the antiglobulin test may be extended to a period of from 60–75 minutes.

If, in addition, a third test is required, this should be the serum-albumin test preferably reading the results by the sedimentation technique after an incubation period of two hours. In our experience the serum-albumin test will very seldom show anything which would not be equally well detected by the antiglobulin and saline agglutination methods, and it certainly cannot be considered in any sense suitable as a substitute for either of these. Its value, as an additional test, is in cases of difficulty or where antibodies are known to be present. It may occasionally demonstrate the activity of anti-M or anti-P at 37° C. Whilst the antiglobulin test is a most valuable test if correctly performed, it is a test whose accuracy is dependent on rigid adherence to the correct technique and unsatisfactory Coombs reagent, inadequate washing of the cells and so forth, are always liable to lead to false negative results in inexperienced hands. If, therefore, there is any doubt as to whether the test is being performed with the requisite care it would be wise to include a serum albumin test in one's cross-matching procedure until such time as one's antiglobulin technique is beyond reproach.

The actual method of carrying out the cross-matching test is as follows:

1. Place the sample of the patient's blood in the space provided in the cross-matching block.
2. Write the patient's name on the cross-matching block.
3. Prepare tubes of separated patient's serum and twice washed cell suspension, placing these in the appropriate holes of the

to take account of the presence of such antibodies. They often occur alone and are not difficult to deal with, and compatible blood is usually not difficult to find. The only difficulty arises when multiple antibodies are present in the same serum, and in these circumstances it might be necessary to disregard one which is active at low temperature alone.

Errors in ABO grouping of donor and recipient should always be picked up in the cross-matching test, using a suitable technique.

CASE 42

Mr. H., a patient suffering from haematemesis, was Group O [illegible] [illegible] positive, the original grouping having been wrongly performed.

CASE 43

Mrs. B.S. was typed as group B under emergency conditions and was known to be Rh positive from previous antenatal testing. She received 500 ml. of group B Rh positive blood and had a severe haemolytic reaction. No cross-matching test was performed, the patient was subsequently regrouped and found to be group A Rh positive. The original ABO grouping had been done on a slide and it was considered that it had inadvertently been turned upside down during the grouping.

The first of these two cases illustrates an original error in the ABO typing detected by the cross-matching test, and the second case shows the error passing undetected when no cross-matching test was performed.

Even subgroups of A, such as A_4, where the blood is thought to be of group O, would normally be found.

CASE 44

Patient Mr. H., group O Rh positive, suffering from haematemesis due to carcinoma of the stomach. It was decided to transfuse him with 2 units of 500 ml. of blood. Both these were group O Rh positive, and a cross-matching test was performed in the usual way, and in the case of the first bottle incompatibility was detected, but not in the case of the second one. Examination showed that the first group O blood was really group A, sub-group A_4.

In the following cases Rh antibodies were involved.

CASE 45

Mrs. A., suffering from severe anaemia, was admitted for treatment by blood transfusion. She was grouped as A Rh positive, and two pints of group A Rh positive blood were cross-matched, found to be

might be immunized as a result of pregnancy: i.e. it could be restricted to people who might show some evidence that they had been immunized. This again, however, is an unsatisfactory premise, firstly, because it is known that intramuscular injections of blood will cause the production of antibodies, especially of Rh antibodies in susceptible persons, and coupled with this it is a fact that persons are often unaware, or have forgotten, that they have received intramuscular injections of blood. A second objection to this is that certain antibodies, anti-Lewis in particular, together with anti-P and anti-M, may occur in persons (both children and adults) who do not appear to have been immunized by previous parenteral injection of blood. Some of these antibodies are known to cause quite severe haemolytic reactions. These considerations show that the cross-matching test is undoubtedly necessary. The most unfortunate premise of all here is that the ABO and Rh typing is always correct; this is not so, and this is one of the main reasons why the cross-matching test is essential.

PURPOSE OF THE CROSS-MATCHING TEST

The purpose of the cross-matching test is to avoid incompatibility and to ensure the best therapeutic results of blood transfusion. This involves:

(*a*) Avoidance of ABO and Rh grouping errors in donor and recipient.
(*b*) The detection of blood group antibodies of various specificities, titres and degrees of avidity dangerously active against donor red cells.

We have said previously in the introduction that the purpose of a cross-matching test is to avoid incompatibility and to ensure the best therapeutic results. It is insufficient to try to produce a technique which will merely avoid frank incompatibility, accompanied by jaundice and anuria, and to ignore lesser degrees of destruction of red cells, because [illegible] and perhaps with a different clinical state, cause a severe and frank haemolytic reaction. In other words, one has to consider the patient as a whole and not merely the antibody. An antibody should not be considered as of little importance simply because it does not always cause a severe haemolytic reaction. Therefore, one cannot distinguish between two desires—that of avoiding incompatibility and ensuring the best therapeutic result; they really amount to the same.

No antibody active at 37° C. can ever be ignored. The question arises whether antibodies which do not act at 37° C. can be disregarded. Here we have found it a useful working rule, and indeed a simple one,

to take account of the presence of such antibodies. They often occur alone and are not difficult to deal with, and compatible blood is usually not difficult to find. The only difficulty arises when multiple antibodies are present in the same serum, and in these circumstances it might be necessary to disregard one which is active at low temperature alone.

Errors in ABO grouping of donor and recipient should always be picked up in the cross-matching test, using a suitable technique.

CASE 42

Mr. H., a patient suffering from haematemesis, was Group O Rh positive, and was cross-matched against supposedly group O Rh positive blood. The cross-matching test showed incompatibility and the donor's blood was thereupon regrouped and found to be group A Rh positive, the original grouping having been wrongly performed.

CASE 43

Mrs. B.S. was typed as group B under emergency conditions and was known to be Rh positive from previous antenatal testing. She received 500 ml. of group B Rh positive blood and had a severe haemolytic reaction. No cross-matching test was performed, the patient was subsequently regrouped and found to be group A Rh positive. The original ABO grouping had been done on a slide and it was considered that it had inadvertently been turned upside down during the grouping.

The first of these two cases illustrates an original error in the ABO typing detected by the cross-matching test, and the second case shows the error passing undetected when no cross-matching test was performed.

Even subgroups of A, such as A_4, where the blood is thought to be of group O, would normally be found.

CASE 44

Patient M[illegible]
due to carc[illegible]
with 2 uni[illegible] ml. of blood. Both these were group O Rh positive, and a cross-matching test was performed in the usual way, and in the case of the first bottle incompatibility was detected, but not in the case of the second one. Examination showed that the first group O blood was really group A, sub-group A_4.

In the following cases Rh antibodies were involved.

CASE 45

Mrs. A., suffering from severe anaemia, was admitted for treatment by blood transfusion. She was grouped as A Rh positive, and two pints of group A Rh positive blood were cross-matched, found to be

compatible and given to her. Three months later she was readmitted for a second transfusion and on the second occasion regrouped as group A Rh positive and the cross-matching test with homologous blood was set up and found to be incompatible. Subsequent investigation showed that the patient was, in fact, group A Rh negative, and had Rh antibodies in her serum.

CASE 46

Mrs. H., aged 30, was admitted to hospital, with incomplete abortion accompanied by haemorrhage. She was suffering from shock and her haemoglobin was 8·5 grammes per cent. The patient was grouped A Rh positive, and given one pint of group A Rh positive blood (R_1r). During the transfusion she developed a severe rigor. She was subsequently found to be jaundiced and had haemoglobinuria followed by anuria, but made an uninterrupted recovery. Investigations showed that the patient was group A Rh negative and that her serum contained Rh antibodies (anti-D).

These last two cases illustrate the points previously made. It is shown in Case 45 that the cross-matching test was not able to prevent immunization of the recipient where a mistake had been made in the Rh group, and was only able *to show that it had occurred and to prevent an incompatibility when the patient had developed Rh antibodies in her serum. In Case 46* the patient's Rh group was wrongly done and she had antibodies in her serum, but unlike the first case, they were not detected in the cross-matching test and consequently the error was not discovered and the patient had a severe haemolytic reaction.

CASE 47

A child, aged 5 years, received severe burns and was admitted for a skin grafting operation. The child was grouped as group O Rh positive and given group O Rh positive blood. The child made a good recovery, went home, and returned six months later for further skin grafting. The child was again grouped as group [illegible] positive, and a cross-matching test set up with [illegible] e blood. Incompatibility was [illegible] l was in fact [illegible]

Th[illegible] shows again that immunization could [illegible] *by the cross-matching test, but was detected when it had occurred. The interest in this case lay in the fact that such immunization can occur in young children and consequently similar* precautions to those employed *in the case of adults* should be exercised in these cases.

In the next three cases, ABO incompatibility occurred, and a pint of whole blood was given to each of these three patients. They were all suffering from different conditions and were different clinically, and greatly different results were produced. In the first case incompatibility

was followed by severe shock and death, although in this case it must be admitted that there had been some shock originally. In the second case, the patient had no jaundice but had a rigor followed subsequently by anuria, and in the third case no symptoms were observed. These three cases illustrate the different reactions of patients to incompatible transfusion.

CASE 48

Mr. A., admitted to hospital following an accident, resulting in a fractured femur. The patient was not severely shocked but it was considered before operative treatment was started that he should receive a pint of blood. The patient was grouped as O Rh positive, and inadvertently was given a pint of group A Rh positive blood. No cross-matching test was performed. Severe shock occurred following the transfusion and the patient died six hours later.

CASE 49

Mrs. H., aged 48 years, was admitted to hospital with severe menorrhagia and anaemia. Hysterectomy was intended. She was group O Rh positive, and had already received 1 pint of group O Rh positive concentrated red cells when she was accidentally given 1 pint of group A Rh positive whole blood. The patient had a severe rigor, but was not shocked or collapsed, and there was no jaundice. She later developed anuria, but made a good recovery following suitable treatment.

CASE 50

Blood was being cross-matched for two patients at the same time; one was group O Rh positive and the other group AB Rh positive. The cross-matching tests were carried out satisfactorily and were compatible, but unfortunately the compatibility labels were stuck on the wrong bottles, so that each patient received the blood intended for the other. Fortunately no ill effects followed.

In all these cases of transfusion reaction, in which there was destruction of blood, the destruction was attributed to the giving of incompatible blood. In order to establish this with certainty, however, it would have to be demonstrated that the transfused cells were abnormally rapidly removed from the circulation, and that no other cause was present to account for the signs and symptoms that occurred. It was not possible to demonstrate this in all these cases, but in many of them the evidence was such that the cause of the haemolysis was considered to be the incompatible transfusion. In some cases [illegible] [illegible] [illegible] [illegible] haemoglobinuria or other sign or symptom of incompatible transfusion had resulted.

Assessment of the Value of the Cross-matching Method

Lastly, we come to the assessment of a particular cross-matching method, i.e. its value in clinical practice. Assuming the techniques employed in the method are satisfactory (p. 221) are there any other means by which the value of the cross-matching method can be determined during its use in clinical practice?

One way is to try to analyse the cases which have been transfused in a hospital in order to determine whether any incompatible transfusions have occurred during a certain period of time. It will not have been possible, of course, unless detailed investigations have been done, to detect abnormally rapid removal of cells from a patient's blood as distinct from cases which show frank signs and symptoms of clinical haemolytic reaction. However, it is important to ascertain the number of such clinical haemolytic reactions which are due to haemolysis of blood in vivo and which are attributable to technical errors in the cross-matching method.

Another assessment that may be made is to determine the number of incompatibilities that have been detected during the performance of cross-matching tests in the laboratory. That is to say, the pathologist may find that in 1,000 consecutive cross-matching tests no incompatibilities have been detected using a particular technique. This may well be regarded as due to correct ABO and Rh grouping and the absence of atypical antibodies in the recipients' sera. To some extent this may be true and it will depend upon the particular type of case for which blood is being supplied and whether any cases are regularly being transfused that have received multiple transfusions, as well as the age of the stored blood normally used. Nevertheless it would be unusual if in 1,000 cases, incompatibilities of some kind were not detected, since experienced workers do find them in such a series of cases. Love and Wallace (1956) found 208 incompatibilities in cross-matching blood for 20,000 patients. This, of course, does not refer to incompatibilities which have resulted from procedural or clerical errors, but from those which have been detected as resulting from ABO and Rh grouping or the occurrence of atypical antibodies. Of a sample 1,000 cross-matchings undertaken at the Manchester Royal Infirmary only three incompatibilities (all due to the presence of atypical antibodies in the recipients' sera) were detected during the performance of these tests and no haemolytic transfusion reactions occurred in the hospital during this time (Lempert, 1958).

Analyses of this kind in a hospital, although not of very great value in assessing the cross-matching method, may be a useful addition to the knowledge that suitable techniques are used which will detect a high proportion of blood group antibodies (*vide infra*). On the other hand, it cannot be argued that a cross-matching method is satisfactory on the

grounds that no incompatibilities have been detected in a large series of consecutive tests, because this might prove just the opposite, namely, that the technique used is insufficiently sensitive.

Factors Governing the Selection of Technique in the Cross-matching Test

These may be tabulated as follows:

1. Experience.
2. Sensitivity of the technique in the detection of:
   (*a*) Anti-A, anti-B.
   (*b*) Other blood group antibodies.
3. Test should be able to be conducted with reasonable speed.
4. Test should be relatively easily performed and not be unduly complicated.
5. Test should be reliable, giving a minimum of false results.

Experience of surveys of incompatible transfusion reactions, and of surveys of difficulties encountered during the cross-matching test, shows that the greatest number of haemolytic reactions are due to ABO incompatibility between donor and recipient. Since anti-A and anti-B occur normally and regularly in the sera of recipients, it is important that the cross-matching test should include a technique which will detect such antibodies regularly and easily. This technique will, of course, if properly arranged, detect other blood group antibodies which act as saline agglutinins, but the detection of anti-A and anti-B must be its primary purpose. Whilst very speedy and accurate methods can be employed to detect anti-A and anti-B these are usually not so sensitive in the detection of other saline agglutinins and, therefore, one has to try to find a technique which enjoys the best of all worlds, and at the same time is sufficiently sensitive to detect weak anti-A and anti-B. We have never seen anti-A totally absent from a serum, where it would be expected, but have seen it greatly reduced in activity and sometimes resembling an anti-A_1. Anti-B, also, is sometimes only very weakly active. One part of the cross-matching test, therefore, should be a saline agglutination test carried out in a tube at room temperature (or 16° C.). The test is designed in this way for the following reasons:

1. A tube technique is employed since this is more sensitive and convenient than a method using slides or tiles.
2. A saline medium is selected because anti-A and anti-B are preferentially detected in a saline medium.
3. Since as many as 2–3 per cent of anti-A or anti-B antibodies may fail to show decisive agglutination at 37° C. it is necessary to carry out the test at some lower temperature; 16° C. is selected as a temperature which will give a strong agglutination with anti-A

and anti-B without being so low as to cause frequent interference from undesirable cold autoantibodies.

4. Cold specific atypical antibodies such as anti-P and anti-M will also readily be detected by this test.

Anti-A and anti-B are readily detected after a very short period of incubation of the cells and serum, if the mixture is centrifuged. However, such a procedure would make it difficult to detect Lewis antibodies and antibodies such as anti-P, and therefore a preliminary period of incubation of 40 minutes coupled with centrifugation, is suggested as a technique to pick up the maximum number of saline agglutinins and preferentially detect anti-A and anti-B. Alternatively the saline agglutination tests can be read by the sedimentation technique after 1½ to 2 hours incubation.

A saline agglutination test at 37° C. is also included because it is not only of assistance in diagnosing false positive results which may occur at 16° C. but also because certain antibodies will be detected at this temperature. Lewis antibodies are the most important here because of their frequency, and liability to cause haemolytic reactions.

It is known that certain techniques, such as "layering" techniques, will readily detect saline agglutinins of certain specificities—for example, those present in anti-Kell sera—but such techniques are disadvantageous for the detection of anti-A and anti-B and consequently not recommended for use in the cross-matching test.

A further technique is needed for the detection of incomplete antibodies, and we recommend one technique only, rather than two. This is done on the grounds of speed, ease of performance and simplicity of the test, and it therefore remains to consider which of three possible tests should be selected. These are: the papainized cell technique, the albumin or serum albumin method, or the antiglobulin test.

Dealing first with the papainized cell test, it may be said that this has the advantage of speed and simplicity. It is a very sensitive means of detecting anti-A, anti-B, anti-P and Rh antibodies of all types, and it will also detect Lewis antibodies, but it fails to detect anti-M, anti-N, many anti-K, anti-S, anti-Fy^a and anti-Jk^a. The papainized cell technique is a method primarily designed for the detection of antibodies in sera using a bulk supply of carefully prepared and carefully controlled papainized cells for testing a large number of different sera, and it is much less suitable if used by papainizing the cells of a number of different donors in order to test these against a patient's serum. The reagents needed to papainize the cells are rather unstable and no cells can be considered satisfactorily papainized until they have been standardized (p. 40).

This makes the test a lot less simple than would at first sight appear to be the case. Furthermore, some cell samples seem to be less easily

papainized than others, and we have met with at least two cell samples which seemed most difficult to papainize, or indeed, to change by any other enzymes. A further disadvantage of the papainized cell test is that it is so sensitive as to detect a large number of weak autoagglutinins, weak anti-H/O and anti-P active only against papainized cells. Most of these are so weak that it is almost certain that they are not of any clinical significance so that their detection is merely a nuisance. In short, this technique is not recommended for cross-matching.

The albumin, or serum albumin test, may be employed as a second test but suffers from certain disadvantages. It fails to detect certain antibodies that the antiglobulin test will detect, e.g. certain Rh antibodies, anti-Fy^a and anti-Jk^a. Moreover, it is a test which is subject to zoning, which is a clear disadvantage in any technique where simplicity is the order of the day, and a titration method would be laborious. Also, the albumin test, especially after centrifugation, is a difficult test to interpret.

The antiglobulin test, on the whole, is considered to be the test of choice. It is widely effective in picking up all kinds of incomplete blood group antibodies, and provided that the technique and reagents are as laid down in Chapter IV, and the special precautions which are suggested therein are followed, no difficulties will be encountered in using this method. It can be completed within the hour. Zoning is very rarely a cause of difficulty, but the antiglobulin reagent can readily be diluted and the test repeated if it is considered desirable to do this. Furthermore, the test is one which is very applicable to cases where rouleaux formation occurs.

It is also by and large the test which shows the best correlation with the clinical importance of the antibodies, and, in general, antibodies which are active in the antiglobulin test are dangerous.

Cross-matching Difficulties

When positive results are encountered in the cross-matching test, one must first of all try to decide whether they are true positive results (which will usually be due to the action of a specific blood group antibody) or false positive results. This is the reason for including the tests of the patient's serum against his own cells.

False Positive Results

Whilst any of the false positive results mentioned in Chapter V can, of course, occur in a cross-matching test, the following will be specially considered here:

1. Rouleaux formation.
2. Autoagglutination.
3. Infection of serum, or red cell suspension.

4. Clots.
5. Direct positive antiglobulin test on donor's cells.

1. ROULEAUX FORMATION. When this occurs in saline agglutination tests, it can be recognized microscopically, or at least suspected, and disappears in the majority of cases when the serum is diluted 1 in 2 in saline. If time permits, therefore, the saline agglutination test, in which it is thought that rouleaux formation occurs, may be repeated using the recipient's serum diluted 1 in 2 in saline. Certain pathological conditions, such as multiple myelomatosis, and other abnormalities of the serum proteins, result in very marked rouleaux formation, and here definite aggregates of cells can be seen which resemble true agglutination. It is important to note that the cell aggregations due to rouleaux formation will occur in the control mixtures of the patient's own cells and own serum in saline at room temperature and at 37° C. Rouleaux present difficulties in cases where true agglutination is present in the serum at the same time as the rouleaux forming properties. The agglutinin may have a titre greater than 1 in 2 and in such circumstances it should be possible to distinguish it from the presence of the agglutinin by comparison with the control. The antiglobulin test is particularly valuable in this respect since it is not affected by the presence of rouleaux forming properties in the serum.

Summarizing, therefore, if rouleaux formation is suspected, the following steps should be taken:

(*a*) Inspect the control, patient's own cells and own serum, which will give a similar appearance at room temperature and at 37° C.
(*b*) Have experience of microscopic examination of rouleaux formation which will help in the diagnosis (Plate IX).
(*c*) If time permits, dilute the patient's serum 1 in 2 and repeat the tests.
(*d*) Read the antiglobulin test which is not affected by rouleaux forming properties of the serum.
(*e*) Remember that agglutinins may be present in serum that contains rouleaux forming properties. These usually have a titre greater than 1 in 2 but this may not be universally so.

2. AUTOAGGLUTINATION. Cold agglutination, as its name implies, occurs at temperatures below 37° C. and is of two kinds: specific and non-specific. The non-specific type is called autoagglutination and presents the following characteristics:

(*a*) *It is present in the saline agglutination test at room temperature; not present in the saline agglutination test at 37° C.*
(*b*) *It is present in the control tube (patient's own cells and own serum) at room temperature but not at 37° C.*

(c) It may appear to be active at 37° C. as well as at 16° C. if care is not taken to prevent cooling whilst reading the tests.

When autoagglutination is present the first step is to ensure that this has not led to errors in the determination of the patient's ABO and Rh groups:

(i) Prepare a free cell suspension of the patient's red cells by washing three times in saline. In cases where the autoantibody is a high temperature one, the cells will need to be washed in saline at 37° C. In certain special cases, such as cases associated with acquired haemolytic anaemia, it may be necessary to wash the cells at higher temperatures still in order to ensure that they are freely suspended in saline (p. 246).

(ii) Washed cells are placed in tubes at 16° C. and 37° C. with anti-A, anti-B, AB serum and saline. The patient's serum is put up with A, B, and O cells at 16° C. and at 37° C. also. The results are read microscopically. The tests sometimes give stronger results with group O than with group A or B cells and this may suggest the presence of a specific antibody in addition to the autoagglutinin, though certain autoagglutinins will themselves give this pattern (see p. 93).

(iii) Test the washed cells with anti-D by the tube technique at 37° C. using an AB serum control.

The cross-matching test can then be carried out taking the precautions against cooling mentioned on p. 92. That an agglutinin is an autoagglutinin and not a specific blood group antibody would have to be ascertained finally by absorbing the serum with the patient's own cells previously washed free from antibody at low temperature, and demonstrating that this removes the agglutinin to the other red cells. Such procedures are not usually possible in an investigation of the cross-matching test.

3. INFECTION. Infection is an occasional cause of false positive results occurring in the cross-matching test. The red cells from the bottle of blood or side tubes should be sterile. It is only when side tubes have been opened several times that contaminated red cells are liable to be used, and under such circumstances if contaminated red cells are used it will be fo[illegible]

serum anc[illegible]

present at [illegible]

[illegible]

4. CLOTTING DIFFICULTIES. These often cause trouble. It may happen that the suspension of donor's cells contains small clots. This occasionally occurs in samples obtained from the bottle of blood or from side tubes, especially if the blood has not been thoroughly mixed with the citrate solution during its collection. These small clots may consist of ten, twelve or more cells and look very like an agglutination, but experience will often enable them to be recognized since they have a characteristic appearance. If it is suspected that clots are present, the cell suspensions should be stood on the bench for a few minutes to permit these to sink to the bottom of the tube and the supernate removed. Indeed, this is a useful precaution to follow in all cases; that is, to have a long tube 3 × ½ inch, and permit the cell suspension to stand in it and to obtain the cell suspensions for the test from the top half of the tube.

Sometimes difficulties occur if the donor's cell sample has been obtained from the bottle of blood, and not been sufficiently washed free from plasma. If these partially washed cells are mixed with the patient's serum, clotting will occur, especially when the mixtures are stood at 37° C. and this may mimic agglutination. The strands of fibrin may enmesh the cells or cause them to clot together so that an appearance of false agglutination is seen. Watch should be kept for this phenomenon and the donor's cells sufficiently washed to be free from plasma so that this difficulty will not occur.

Sometimes difficulties occur due to the patient's serum. If a cross-matching test has to be done rapidly and the patient's serum is separated from the cells by centrifugation before clotting is complete, clotting may continue in the cross-matching test and may enmesh the cells and cause an appearance of agglutination. This is most likely to occur when clotting defects are present in the patient; for example, in cases of haemophilia.

5. DIRECT POSITIVE ANTIGLOBULIN TEST ON DONOR'S CELLS. If the direct antiglobulin test on the donor's cells is positive then the donor's blood is unsuitable for use as transfused blood. A number of cases of this may be due, of course, to the donor having an acquired haemolytic anaemia of some kind, although this is unlikely. Alternatively, it may be due to the fact that it is a direct positive antiglobulin test either with anti-γ-globulin reagent, or in some cases anti-non-γ-globulin reagent, unassociated with any signs or symptoms in a perfectly healthy donor (Chapter IV).

TRUE POSITIVE RESULTS

If it is concluded that the difficulty is not due to false positive results or that both false and true positive results are occurring, it is presumed that a specific blood group antibody is present, and steps should be taken to identify it. The first thing to remember

here is that the ABO or Rh groups of patient or donor may be incorrect, and if this is suspected these groups should be carefully retested. Very rarely the supposed group O donor is, in fact, group A_1. Sometimes it is found that the patient's blood has been wrongly grouped as D positive when, in fact, it is D negative with anti-D in the serum.

If the ABO and Rh groups are correct, the results fall roughly into two classes:

(*a*) those where the saline agglutination test at one or the other temperature is positive, and
(*b*) those where the saline agglutination test is usually negative, but may be positive, and where the antiglobulin test is positive.

It is undesirable at this stage to proceed forthwith to the screening of bottles, and hope without establishing the identity of the antibody to find sufficient compatible bottles for the patient's needs. This is a bad practice since, in the first place, nothing is ever learned about how the antibodies react, how they are detected, and which are dangerous antibodies. Secondly, it is not safe to proceed and give an apparently compatible blood if another is incompatible, without diagnosing the nature of the antibody. The reasons for this are that the antibody may be difficult to detect unless special care or techniques are used; for example where anti-Le^a+anti-Le^b are present in the same serum, the cross-matching test perhaps having only detected the anti-Le^a. The routine cross-matching test is intended for the general detection of antibodies of all types, but does not necessarily include the most sensitive method for every individual antibody. Once an antibody has been detected the case becomes a special case, calling for special care, and possibly for special serological methods according to the particular antibody involved. This is illustrated by Case 70, p. 265. Sometimes the antibody is detected using homozygous cells, so that the strong reaction under these circumstances is detected, but heterozygous cells, which under more sensitive tests would react with the antibody, are wrongly thought to be compatible.

CASE 51

Mrs. G., group O rr, was found during the antenatal period to have strong incomplete anti-D in her serum. When her baby was born it was found to be group AR_2r. The direct Coombs test was strongly positive, the haemoglobin 13 gm. per cent and the serum bilirubin 4·8 mgm. per cent. Two bottles of group A Rh negative blood were cross-matched against the infant's serum without difficulty, and an exchange transfusion carried out, but the serum bilirubin continued to rise and by the following morning had reached a level of 17·0 mgm. per cent. A second exchange transfusion, therefore, was advised. Cross-matching tests using the infant's serum were carried out with four bottles of A Rh negative blood.

Two of these were found incompatible by the indirect Coombs test, but the other two were found compatible and were used for a further exchange transfusion. Following the second exchange transfusion, the infant's serum bilirubin was 12·6 mgm. per cent, and it showed signs of kernicterus. The infant died the following evening and kernicterus was confirmed at the post-mortem. Samples of the mother's blood, the infant's blood and the two bottles of blood used at the second exchange transfusion were referred to us for further investigations, when it was found that both the mother's and the infant's sera contained a potent anti-A of immune type detectable with anti-non-γ-globulin type reagents and with cells suspended in serum albumin. A series of direct Coombs tests on various samples of the infant's blood gave the following results:

	Anti-γ-globulin	*Anti-non-γ-globulin*	*Saline*
At birth	++++	w	—
Before 1st exchange transfusion ..	++++	w	—
After 1st exchange transfusion ..	+++	++	—
Before 2nd exchange transfusion ..	+++	++	—
After 2nd exchange transfusion ..	++	+++	—

It seems that the cause of the deterioration in the infant's condition was the reaction of the immune anti-A in its serum with the transfused adult A cells and it is interesting to notice that although the direct Coombs test with anti-γ-globulin reagent became weaker following the successive exchange transfusions with Rh negative blood, the direct Coombs test using the anti-non-γ-globulin reagent became progressively stronger. This appears to be due to the greater reactivity of the adult A antigen with immune anti-A. The remains of the two bottles of blood used in the second exchange transfusion were now examined and it was found that bottle "1" was group A_2 and bottle "2" group A_1. We repeated the cross-matching tests using the infant's serum and obtained the following results:

	Antiglobulin test		*Serum albumin*	*Saline*	
	Anti-γ	*Anti-non-γ*	*37° C.*	*37° C.*	*16° C.*
Bottle 1 .. .	—	w	++	—	—
Bottle 2	+	+++	+++	—	—
Baby's cells ..	++++	w	+++	—	—

In this case an incompatibility was detected in the cross-matching test, but other bottles of blood which appeared to be compatible were given with deleterious effects. The case showed that if one bottle is found incompatible in a cross-matching test *it is an indication that this is a difficult case and one where special care is needed*

in cross-matching other bottles. The case also illustrates the value of an anti-non-γ-globulin reagent, which showed very clearly what had happened to the child following the successive exchange transfusions. This type of reagent was also of considerable value in the elucidation of the cause of the difficulty. The repeat cross-matching test showed that bottle "1" was completely compatible using an anti-γ-globulin reagent and bottle "2" was only weakly incompatible. This was also one of those rare cases where the serum albumin test was of value in cross-matching, and if it had been employed in the first place it would have shown that these two bottles were incompatible.

CASE 52

A patient suffering from a severe haemorrhage following caesarian section was found to be group B Rh positive, and a cross-matching test was carried out with four group O Rh positive bottles of blood, since no group B blood was immediately available. One of the bottles was incompatible by the indirect Coombs test, but the other three appeared to be compatible and were given to the patient. She developed a severe haemolytic transfusion reaction, became jaundiced, and died some hours later from the effects of haemorrhage, hepatic necrosis and the transfusion. Subsequently, a direct Coombs test showed clearly the presence of incompatible transfused cells in her circulation (Plate XVb) and an incomplete anti-c, active in the Coombs test, was found. This gave a marked dosage effect, and it is likely that some of the bottles given to the patient were heterozygous C-c, and that their incompatibility was overlooked for this reason.

The conclusion here, therefore, is that if incompatibility occurs in the cross-matching test, any compatible bottles should be considered to be only apparently compatible until the nature of the antibody is determined. Another thing that must be remembered is that when one antibody is present others may be present also. This is particularly important where a patient has been receiving multiple transfusions and where the first antibody was originally detected a long time ago and compatible blood has since been given.

The identification of the antibody is also of importance because, if it is active at room temperature only, it may be wondered whether it is a dangerous antibody or not. Mollison (1957) has stated that antibodies which are active only below 31° C. are not dangerous, but for the purpose of the technique described herein we have considered that all antibodies detected at 16° C. and higher might be potentially dangerous. It is often very difficult to say whether such antibodies have a small activity above 31° C. or even whether a very feeble reaction is given at 37° C., and if circumstances permit, as they most often do, it is better to give compatible blood.

The rule, therefore, is that when incompatibility occurs the antibody

should be identified before proceeding further. Whilst it is very desirable to keep to this rule whenever possible, it has to be conceded that cases do occur in which one has to be content with the empirical method of cross-matching a number of bottles, and giving those which are found compatible in the tests. This may arise from the urgency of the case; or because difficulties are encountered in attempting to identify the antibody, or from other adverse circumstances. Cross-matching in this way is a departure from the ideal, and will only be safe if carried out observing all the precautions described in other chapters. Moreover, it does not always effect a saving of time or labour, since it may be that when a patient's serum is cross-matched against a large number of bottles of blood, few or none will be found to be compatible. Whereas, if the antibodies are identified first it may well be that donors of suitable groups will be known already. When this method does have to be used an attempt ought always to be made to identify the antibody subsequently in case further transfusions are required.

In identifying the antibody present, the results obtained in the initial incompatible cross-matching test provide a valuable clue, and one should consider whether the antibody is active only in the saline agglutination test and, if so, at what temperature, or whether it is active in the Coombs test. If several bottles of blood have been cross-matched, one should also consider whether all or only some were found incompatible. Table 60 shows the commonest patterns of results obtained in the different tests, together with the antibodies that are most likely to be causing the difficulty.

TABLE 60. MOST LIKELY ANTIBODY ACCORDING TO THE DIFFICULTY IN THE CROSS-MATCHING TEST, ASSUMING THE ABO AND RH TYPING TO BE CORRECT

Antiglobulin test	*Saline agglutination test*		*Most likely antibody*
	37° C.	16° C.	
neg.	neg.	pos.	Anti-A_1, M, N, O, H, P, Le^a, Le^b
neg.	pos	pos.	Anti-Le^a, Le^b Rarely anti-M, P.
pos. Anti-γ-globulin reagent	pos. or neg.	pos. or neg.	Anti-E, c, D*, K, Fy^a.
pos. Anti-non-γ-globulin reagent	pos. or neg.	pos. or neg.	Anti-Le^a, Le^b, Jk^a.

* If recipient's Rh group has been incorrectly determined.

When these points have been considered, the antibody may be identified, as described in Chapter XII. When this has been done, blood lacking the antigen concerned should be provided for the cross-matching test. It may not always be possible to do this, especially in cases of urgency, and if the antibody in the patient's serum is sufficiently potent it may be possible to rely on this. If, however, it is weak in its activity, it will not be possible to rely on it, but it might be possible to get around the difficulty. For example, if an R_1r patient had anti-E in the serum, cross-matching could be carried out with Rh negative blood, though if the patient is R_1R_1 this is inadvisable since we have known the transfusion of Rh negative blood to an R_1R_1 patient with anti-E in the serum result in the development of a potent anti-c.

Anti-Le^a on its own and anti-Le^b on its own present some difficulty, but in combination they present even greater difficulty because even certain cells which are known to be type Le(a−b−) may prove to be incompatible. It is not known at present whether these incompatible Le(a−b−) cells are more likely to be destroyed in vivo than are compatible Le(a−b−). The Le(a−b−) cells which are compatible are often those which come from persons who are non-secretors of Lewis substances. These non-secretors are rare and difficult to find, and if it is not possible to find them then one might have to resort to transfusion with Le(a−b−) cells from persons who are secretors. Lewis antibodies in a patient's serum can sometimes be used for selecting blood by using their haemolytic activity, and the bloods which fail to give lysis are then further examined by use of the saline agglutination technique with centrifugation, and the antiglobulin technique using anti-non-γ-globulin reagents.

It may be that more than one atypical antibody is present in the serum of a patient needing transfusion, and these will all have to be identified by using the standard cells and the methods described for the identification of atypical antibodies. A good guide to the nature of those antibodies can be had from a knowledge of the frequency of their reactions and the nature of their reactions, as shown in other chapters.

Difficulties arise when the controls as well as the actual tests are positive. This often occurs in cases of symptomatic and idiopathic acquired haemolytic anaemia and these are special cases which will be dealt with later, as are high temperature autoantibodies. Rouleaux and or autoagglutination can occur in combination with atypical antibodies and under the latter circumstance it might be found that the control test with the patient's own cells and own serum is positive at 16° C. but not at 37° C., whereas the cross-matching test is positive at both temperatures. These do not usually present the difficulties that they seem to present from the text and are usually readily resolved if the instructions in Chapter V are followed.

The procedure described is more intended for guidance than for rigid

should be identified before proceeding further. Whilst it is very desirable to keep to this rule whenever possible, it has to be conceded that cases do occur in which one has to be content with the empirical method of cross-matching a number of bottles, and giving those which are found compatible in the tests. This may arise from the urgency of the case; or because difficulties are encountered in attempting to identify the antibody, or from other adverse circumstances. Cross-matching in this way is a departure from the ideal, and will only be safe if carried out observing all the precautions described in other chapters. Moreover, it does not always effect a saving of time or labour, since it may be that when a patient's serum is cross-matched against a large number of bottles of blood, few or none will be found to be compatible. Whereas, if the antibodies are identified first it may well be that donors of suitable groups will be known already. When this method does have to be used an attempt ought always to be made to identify the antibody subsequently in case further transfusions are required.

In identifying the antibody present, the results obtained in the initial incompatible cross-matching test provide a valuable clue, and one should consider whether the antibody is active only in the saline agglutination test and, if so, at what temperature, or whether it is active in the Coombs test. If several bottles of blood have been cross-matched, one should also consider whether all or only some were found incompatible. Table 60 shows the commonest patterns of results obtained in the different tests, together with the antibodies that are most likely to be causing the difficulty.

TABLE 60. MOST LIKELY ANTIBODY ACCORDING TO THE DIFFICULTY IN THE CROSS-MATCHING TEST, ASSUMING THE ABO AND RH TYPING TO BE CORRECT

Antiglobulin test	*Saline agglutination test*		*Most likely antibody*
	37° C.	*16° C.*	
neg.	neg.	pos.	Anti-A_1, M, N, O, H, P, Le^a, Le^b
neg.	pos.	pos.	Anti-Le^a, Le^b Rarely anti-M, P.
pos. Anti-γ-globulin reagent	pos. or neg.	pos. or neg.	Anti-E, c, D*, K, Fy^a.
pos. Anti-non-γ-globulin reagent	pos. or neg.	pos. or neg.	Anti-Le^a, Le^b, Jk^a.

* If recipient's Rh group has been incorrectly determined.

When these points have been considered, the antibody may be identified, as described in Chapter XII. When this has been done, blood lacking the antigen concerned should be provided for the cross-matching test. It may not always be possible to do this, especially in cases of urgency, and if the antibody in the patient's serum is sufficiently potent it may be possible to rely on this. If, however, it is weak in its activity, it will not be possible to rely on it, but it might be possible to get around the difficulty. For example, if an R_1r patient had anti-E in the serum, cross-matching could be carried out with Rh negative blood, though if the patient is R_1R_1 this is inadvisable since we have known the transfusion of Rh negative blood to an R_1R_1 patient with anti-E in the serum result in the development of a potent anti-c.

Anti-Le^a on its own and anti-Le^b on its own present some difficulty, but in combination they present even greater difficulty because even certain cells which are known to be type Le(a−b−) may prove to be incompatible. It is not known at present whether these incompatible Le(a−b−) cells are more likely to be destroyed in vivo than are compatible Le(a−b−). The Le(a−b−) cells which are compatible are often those which come from persons who are non-secretors of Lewis substances. These non-secretors are rare and difficult to find, and if it is not possible to find them then one might have to resort to transfusion with Le(a−b−) cells from persons who are secretors. Lewis antibodies in a patient's serum can sometimes be used for selecting blood by using their haemolytic activity, and the bloods which fail to give lysis are then further examined by use of the saline agglutination technique with centrifugation, and the antiglobulin technique using anti-non-γ-globulin reagents.

It may be that more than one atypical antibody is present in the serum of a patient needing transfusion, and these will all have to be identified by using the standard cells and the methods described for the identification of atypical antibodies. A good guide to the nature of those antibodies can be had from a knowledge of the frequency of their reactions and the nature of their reactions, as shown in other chapters.

Difficulties arise when the controls as well as the actual tests are positive. This often occurs in cases of symptomatic and idiopathic acquired haemolytic anaemia and these are special cases which will be dealt with later, as are high temperature autoantibodies. Rouleaux and or autoagglutination can occur in combination with atypical antibodies and under the latter circumstance it might be found that the control test with the patient's own cells and own serum is positive at 16° C. but not at 37° C., whereas the cross-matching test is positive at both temperatures. These do not usually present the difficulties that they seem to present from the text and are usually readily resolved if the instructions in Chapter V are followed.

The procedure described is more intended for guidance than for rigid

adherence, and it is often possible by following up a clue to short circuit the procedure and find out the cause of the difficulty quite rapidly. If, for example, the patient is group A or AB and has an antibody in his serum active only at 16° C., it is always worth while to test his cells with anti-A_1. If he is found to be A_1 or A_1B, it is quite likely that the antibody will turn out to be anti-O or anti-H and tests with a series of O and A cells should, therefore, be made. If, on the other hand, he turns out to be A_2 or A_2B, it is quite likely that the antibody involved is anti-A_1. Anti-A_1 will usually give some reaction with cells on a slide, especially if the temperature is low enough, and it may be tested for quite rapidly by putting up the patient's serum with a number of group A bloods in parallel with a known anti-A_1 on slides. If no agglutination occurs at room temperature, the slides should be placed in a refrigerator, and inspected again after a few minutes. Of course, the frequent occurrence of autoagglutinins active at 4° C. may lead to some reaction with the A_2 cells in this test, but, nevertheless, differences in the speed and intensity of the agglutination are often sufficient to indicate the presence of anti-A_1. Anti-P can often be detected by a similar technique, though if known papainized P positive and P negative cells are available this would be a better method. Another procedure that is always useful is to test the patient's serum with any papainized cells which may be available, and if one is fortunate enough to have to hand a good series of papainized cells, whose full groups are known, and if the antibody is one which reacts with papainized cells, it is sometimes possible to identify the antibody in a very short space of time, using the slide technique. The appearance and behaviour of the antibody in the incompatible cross-matching test will sometimes provide a clue which may profitably be followed up; for example, if the cells of one of the donors showed lysis and the others showed a cell chain agglutination at both 16° C. and 37° C., a Lewis antibody would be suspected and it might be possible to show that this antibody was involved by rapid tests with papainized Le(a+) and Le(b+), and Le(a−b−) cells which might show agglutination or lysis. In short, it is often worth while to follow up a clue suggesting the presence of a certain antibody rather than keeping rigidly to a standard procedure, though proper tests confirming the identity of the antibody will have to be carried out later.

In an emergency the question might be asked whether blood which is incompatible in the test can ever be given. All antibodies active at 37° C. are dangerous and the question would only arise in relation to antibodies active in saline at room temperature alone and negative in the antiglobulin test. If it is certain that the antiglobulin test is negative such antibodies will most usually be anti-P, anti-A_1, anti-H/O, and occasional examples of anti-Le^a and anti-Le^b. Blood acting negatively with anti-P and anti-Le^a can nearly always be found, but if not the risk may have to be accepted.

The danger is greatest with anti-Le^a or anti-Le^a + Le^b which can act as haemolysins, can cause severe haemolytic reactions and have sometimes deceptive serological reactions.

The Cross-matching Test in Special Cases

These are as follows:

1. Where more than one atypical antibody is known to be present in a patient's serum.
2. Where the patient's serum causes intense rouleaux formation.
3. Where blood is required for the newly born.

Where more than One Atypical Antibody is Present in the Serum

The fact that more than one atypical antibody is present in a serum should always be suspected in cases where one is present, and difficulties in cross-matching may occur due to the presence of more than one antibody. Particularly is this so if Rh antibodies are present and the combination of anti-D+anti-Kell is not uncommon; other antibodies frequently occur with anti-D, e.g. anti-D+anti-Le^a and anti-Le^b, anti-D+anti-P, anti-D+anti-M, and anti-D+anti-Fy^a. Such cases do not present any serious difficulties because in cross-matching they normally resolve themselves into dealing with one antibody, since one is cross-matching with Rh negative cells. In cases, however, where the Rh antibody is anti-E or anti-c, in combination with another antibody, the procedure is to identify both antibodies (or whatever number there are), and to proceed to test cells lacking the commoner antigen involved. For example, if anti-c is [illegible] who are [illegible] available [illegible] National Panel of Donors), then one would have to proceed by first testing with anti-c to select R_1R_1 donors, and by then testing the selected R_1R_1 donors with anti-Kell. Such cases do not present any serious difficulties, especially when anti-Kell is involved, because of the low frequency of Kell positive blood.

Much more difficult are cases where there are more than two antibodies. Such cases are often associated with acquired haemolytic anaemia, and this condition should be strongly suspected in cases where multiple antibodies or bizarre combinations of antibodies are found. Almost all cases in which we have encountered unusual results in the cross-matching test, which failed to conform to any of [illegible] [illegible] known to develop, or cases of *Cl. Welchii* infection.

In cross-matching cases where there is more than one atypical anti-

adherence, and it is often possible by following up a clue to short circuit the procedure and find out the cause of the difficulty quite rapidly. If, for example, the patient is group A or AB and has an antibody in his serum active only at 16° C., it is always worth while to test his cells with anti-A_1. If he is found to be A_1 or A_1B, it is quite likely that the antibody will turn out to be anti-O or anti-H and tests with a series of O and A cells should, therefore, be made. If, on the other hand, he turns out to be A_2 or A_2B, it is quite likely that the antibody involved is anti-A_1. Anti-A_1 will usually give some reaction with cells on a slide, especially if the temperature is low enough, and it may be tested for quite rapidly by putting up the patient's serum with a number of group A bloods in parallel with a known anti-A_1 on slides. If no agglutination occurs at room temperature, the slides should be placed in a refrigerator, and inspected again after a few minutes. Of course, the frequent occurrence of autoagglutinins active at 4° C. may lead to some reaction with the A_2 cells in this test, but, nevertheless, differences in the speed and intensity of the agglutination are often sufficient to indicate the presence of anti-A_1. Anti-P can often be detected by a similar technique, though if known papainized P positive and P negative cells are available this would be a better method. Another procedure that is always useful is to test the patient's serum with any papainized cells which may be available, and if one is fortunate enough to have to hand a good series of papainized cells, whose full groups are known, and if the antibody is one which reacts with papainized cells, it is sometimes possible to identify the antibody in a very short space of time, using the slide technique. The appearance and behaviour of the antibody in the incompatible cross-matching test will sometimes provide a clue which may profitably be followed up; for example, if the cells of one of the donors showed lysis and the others showed a cell chain agglutination at both 16° C. and 37° C., a Lewis antibody would be suspected and it might be possible to show that this antibody was involved by rapid tests with papainized Le(a+) and Le(b+), and Le(a−b−) cells which might show agglutination or lysis. In short, it is often worth while to follow up a clue suggesting the presence of a certain antibody rather than keeping rigidly to a standard procedure, though proper tests confirming the identity of the antibody will have to be carried out later.

In an emergency the question might be asked whether blood which is incompatible in the test can ever be given. All antibodies active at 37° C. are dangerous and the question would only arise in relation to antibodies active in saline at room temperature alone and negative in the antiglobulin test. If it is certain that the antiglobulin test is negative such antibodies will most usually be anti-P, anti-A_1, anti-H/O, and occasional examples of anti-Le^a and anti-Le^b. Blood acting negatively with anti-P and anti-Le^a can nearly always be found, but if not the risk may have to be accepted.

own serum and is equally intense between the donor's cells and the patient's serum. The clumps of cells are very strong; the typical "piles of coins" may not be seen owing to the intensity of the aggregation, and if the patient's serum is diluted 1 in 2 and an equal volume of cells added, this may be insufficient completely to abolish the rouleaux forming property of the serum, and may mislead one into believing that the rouleaux are agglutinates. Under these circumstances one has to resolve the problem by having regard to the nature of the case and especially when it is one in which the serum protein level or the albumin globulin ratio of the serum is disturbed.

Sometimes the clinical diagnosis may be in doubt and it has been possible on more than one occasion for the diagnosis of multiple myelomatosis to be made during the cross-matching procedure because of the observation of intense rouleaux formation.

CASE 53

Mrs. H., aged 62 years, suddenly became ill with pyrexia and dyspnoea. She was treated with antibiotics and later was fit to return to work. At the time, however, it was noticed that she was pale and she was given anti-anaemic treatment. After this she complained of tiredness and feeling ill, and was admitted to hospital where she was considered to be a case of aplastic anaemia and given blood transfusions. The hospital laboratory reported that before [illegible]

was group OR_1R_2, and no atypical antibodies were detected in her serum. Intense rouleaux formation was observed, but by diluting the serum 1 in 2 compatible blood was provided, though rouleaux were still seen. The [illegible]

[illegible] plasma cells was never found, neither were typical changes found in the peripheral blood, and it was not until X-rays of the skull were taken that the typical translucent [illegible]
discovered. I [illegible]
globulin 6· [illegible]
of ACTH [illegible]
globulin tc [illegible]
her condit [illegible]

It is true that in such cases excessive dilution of the serum may be sufficient to dilute out weak atypical antib [illegible]
can be overcome is by relyin [illegible]
test, of course, is of great v [illegible]
intense rouleaux formation, or indeed, rouleaux formation of any degree.

Where dextran is to be given a sample of blood should be taken

body in the patient's serum, or even where there is only one atypical antibody, it is useful to add at least a serum albumin and, preferably, a papainized cell test, to the antiglobulin test, because sometimes such tests will more competently pick up additional antibodies which may be present and one must take every precaution where one antibody is already known to be present.

In cases where multiple antibodies are present, it may be very difficult to find blood lacking all the corresponding antigens, and it may occasionally be necessary to ignore antibodies which are active only at low temperatures, if this is the only way by which compatible blood can be found. This is, however, a departure from the ideal which, as stated previously, is that *normally blood should be provided which does not react with any antibody present in the serum.*

Certain special instances of the detection of more than one antibody in serum will be dealt with in Chapter XII, but one or two points may usefully be mentioned here. An antibody which is dangerous, and not infrequently occurring in human serum, is anti-Lewis. This may occur in the serum either as anti-Le^a or anti-Le^b, or more commonly as a mixture of the two. In the latter case the serum would agglutinate or react by other means, with about 90 per cent of random group O blood samples. The patients in these cases usually belong to type Le(a−b−) and there is a suggestion that they may be more often group A than group O.

The combination of anti-P with other antibodies often gives a high percentage of positive results, since anti-P itself has a high frequency of activity, but when a combination of such an antibody with another one occurs it is often not difficult to distinguish the fact that two antibodies are present, since anti-P is usually only active at a low temperature and the other antibody may be active at a higher temperature.

Summarizing, therefore, where one antibody is present in a patient's serum and has been found during the cross-matching test, and has been identified, the patient's serum should be tested with cell samples lacking the particular antigen concerned by as many techniques as possible to determine whether additional antibodies are present in the serum. If it is obvious that additional antibodies are present in the serum they should also be identified.

Where the Patient's Serum causes intense Rouleaux Formation

This is almost always confined to certain cases:

(*a*) Following *transfusion with dextran and other plasma substitutes.*
(*b*) *Cases of multiple myelomatosis.*
(*c*) *Cases associated with protein dyscrasia, some with hepatic disease.*

Rouleaux formation is intense between the patient's own cells and

then removed from the centrifuge, retapped, and the process repeated. At the end of this time the cells are removed with a Pasteur pipette, placed on a microscope slide and read under the microscope. This technique will detect the majority of anti-A and anti-B agglutinins.

In using such an emergency method:

1. The standard cross-matching test should also be carried out, firstly, in the hope that it may not prove necessary to give the transfusion so urgently and that the result of the test may be available before the blood is required, and, secondly, so that if incompatibility is detected appropriate action may be taken.
2. Clinicians should be informed of the risks involved in emergency cross-matching techniques.

It is undesirable, if it can be avoided, to give blood without any preliminary ABO and Rh grouping of the patient. If this were to be done, it would necessitate the provision of group O Rh negative blood of safe type. This is not always possible and, if it is to be provided on any scale, would probably make the running of a Blood Bank almost impossible. In such cases, possibly, blood substitutes may be used.

If, in normal hospital practice, cases occur in which blood is urgently required and it is said that time is not available for rapid ABO and Rh typing of the patient, then it is desirable that these tests should be done immediately upon admission of the patient to hospital.

Cross-matching Difficulties in our own Laboratory

Table 61 shows the difficulties encountered in cross-matching blood for 2,967 patients in our own laboratory, and it shows the type of difficulty which is likely to be encountered in cross-matching blood for a large number of patients. It also gives an indication of the relative frequency with which these difficulties occur though the frequency of difficulty in this series is unduly high owing to the fact that these were selected patients. A high proportion of these patients had been referred to us for cross-matching by hospitals, either because the hospital laboratory had [illegible]
or [illegible]
ser[illegible] Furthermore, these patients almost always had more than one
bottle [illegible]
of bloo[illegible]
of bott[illegible] cross-matched is perhaps three or four and sometimes the difficulty recurred each time blood was cross-matched for the patient, whilst sometimes the difficulty was only seen with one bottle of blood. Blood of the same ABO and Rh group as the patient was invariably used (except in cases of haemolytic disease of the newborn), and the tests were carried out by the standard technique described in this chapter, though a serum albumin test was also included in all cases

before this infusion if there is any possibility of a blood transfusion being required.

WHERE BLOOD IS REQUIRED FOR THE NEW-BORN

At birth antibodies present in the child's serum are those which have passed the placental barrier.

It may be reasonably concluded, therefore, that blood which is compatible with the maternal serum will also be suitable for transfusion to the baby. It is not entirely clear what kind of antibody crosses the placental barrier; anti-A or anti-B agglutinins or incomplete Rh antibodies or other antibodies may be present in the cord serum. We have observed anti-H and anti-Lewis in cord serum when these were present in the maternal blood.

It is undesirable to give blood of different ABO group from that of the child because of the possibility of the anti-A or anti-B agglutinins in the blood damaging the child's cells, particularly if a large quantity of blood is to be transfused or exchanged. It is our practice, therefore, in normal cases to group the child for ABO and Rh, and select blood of homologous group, and cross-match it using the maternal serum where possible. Where the ABO groups are such that this is not possible, the child's serum has to be used for the cross-matching tests.

In haemolytic disease of the newborn the same procedure is followed but Rh negative or other special blood is given.

Emergency Cross-matching

The methods so far described have included a standard procedure for the cross-matching test, but sometimes it is necessary to cross-match blood more quickly than this. The incubation times of the saline agglutination tests and the antiglobulin tests can be slightly reduced without materially affecting the number of antibodies that would be detected, but the times given in the standard procedure are the times which are considered to be minimum times having regard to the purpose for which the test is intended. However, if only a short time, say 5–10 minutes, is available in addition to that required for rapid ABO and Rh typing, and securing the necessary quantity of homologous blood, then it seems best to use such time to concentrate on a rapid method of excluding ABO incompatibility, since this is the most likely and most serious error. The technique that is recommended is as follows:

The donor's cells are washed in saline once and made up to a 3 per cent suspension (as for the saline agglutination test). Time will not usually permit the cell suspension to be left on the bench for the clots to sink to the bottom, and this risk must be accepted. One volume of the cell suspension together with one volume of the patient's serum is placed in a tube and mixed together by tapping. The tube is then placed in the centrifuge and rapidly spun at 2,500 r.p.m. for 2–3 minutes. It is

	21	
Anti-P	2	
Anti-Kell	1	
Anti-Kell+anti-D	1	
Anti-M	1	
Anti-Lu^a	1	
Anti-Fy^a	1	
Anti-Jk^a	1	
Antibodies whose identity could not be established (reaction of antibody weak, death of patient, etc.)	25	
Total		114

Acquired haemolytic anaemia and similar conditions

A.H.A. (warm antibody type) with serum containing anti-C	1	
„ „ „ „ „ „ „ anti-c	1	
„ „ „ „ „ „ „ anti-E	2	
„ „ „ „ „ „ „ anti-e	2	
„ „ „ „ „ „ „ anti-C+anti-e ..	2	
„ „ „ „ „ „ „ anti-c+anti-E ..	1	
„ „ „ „ „ „ „ anti-S	1	
(some of the above had a non-specific component also)		
A.H.A. (warm antibody type) with serum containing non-specific antibody only	8*	
„ „ „ „ „ containing little or no free antibody	3	
A.H.A. (cold antibody type) non-specific antibody only	5	
„ „ „ „ serum containing anti-P	1	
Other patients unexpectedly found to have positive direct Coombs test	11	
Donor with positive direct Coombs test	3	
Anti-C+anti-e reacting with patient's papainized cells but direct Coombs test negative and no evidence of blood destruction ..	1	
Non-specific reactions associated with leukaemia	4	
„ „ „ „ syphilis	1	
„ „ „ „ septic abortion (*Cl Welchii*) ..	2	
Total		49

Miscellaneous

Donor's blood not of ABO group stated on bottle	2	
Blood cross-matched against sample from wrong patient	1	
Cause of difficulty not elucidated	13	
Total		16
GRAND TOTAL ..		825

References

Lempert, H. (1958). Personal communication.

Love, Mary, and Wallace, J. (1956). Proceedings of the VIth congress of the International Society of Blood Transfusion, p. 425.

Mollison, P. L. (1956). *Blood Transfusion in Clinical Medicine*. 2nd Ed. [illegible]

[illegible] Oct. 1957.

[illegible] . *Med. J.*, ii, 683.

owing to the high proportion of difficult cases with which we have to deal. The agglutination tests were read by the sedimentation method in most cases though the centrifugation method was sometimes employed.

TABLE 61. DIFFICULTIES ENCOUNTERED IN CROSS-MATCHING BLOOD FOR 2,967 PATIENTS

Errors of technique			
Infected bovine albumin		4	
Unsatisfactory bovine albumin (rouleaux forming)		5	
Chemical contamination of sample		2	
Bacterial contamination of pilot tube		7	
Use of inadequately washed glassware		9	
Clots simulating agglutinates		1	
Total			28
Non-specific effects (patient's serum reacting with patient's own cells)			
Rouleaux affecting the saline and serum albumin tubes of the cross-match	94		
Rouleaux affecting the serum albumin tubes only	244		
Rouleaux affecting the sera with patient's own cells only (no difficulty in actual cross-match)	78		
Total rouleaux		416	
Autoagglutinins (excluding those associated with acquired haemolytic anaemia)		83	
Difficulties associated with use of unsatisfactory Coombs reagents		74	
Total			573
Difficulties with the patient's ABO or Rh group			
Cell mixtures affecting the ABO group		2	
ABO group of patient different from that stated by hospital (leading to doubt regarding correct identity of patient)		5	
Weakness of anti-B in patient's serum in old age		1	
A_4		1	
Cell mixtures affecting the Rh group		31	
D antigen blocked by incomplete anti-D (haemolytic disease of the newborn)		1	
D^u giving weak reactions with agglutinating anti-D sera		1	
Polyagglutinability of patient's cells		3	
Total			45
Specific antibodies in patient's serum			
Anti-A_1		18	
Anti-A_1+rouleaux formation		1	
Anti-A_1+autoagglutinin		1	
Anti-O or anti-H (saline agglutinin)		8	
Anti-c		5	
Anti-c+anti-S		1	
Anti-c+anti-P+autoagglutinin		1	
Anti-E		5	
Anti-Le^a		5	
Anti-Le^b		4	
Anti-Le^a+anti-Le^b		10	

in whom an anti-e antibody occurred. In this case they were able to elute anti-e antibody from the patient's red cells. It is interesting to note that this man had never received any blood transfusions or injections of blood. Similar cases have been described, many Rh and other antibodies being involved, of which the most frequent is anti-e. Case 54 illustrates the findings in a typical case where specific Rh antibodies were present.

Non-specific antibodies are sometimes found, though it may be difficult to decide whether the antibody is non-specific, since more than one antibody may be present. Many of the cases of multiple antibodies present in a patient's serum have occurred in persons suffering from acquired haemolytic anaemia. Callender and Race (1946) found anti-C^w and anti-Lu^a antibody for the first time, as well as the rare anti-Levay antibody, in the serum of a patient suffering from a similar condition. It is also impossible to conclude that the presence of a specific antibody in the patient's serum means that a similar antibody may be eluted from the patient's erythrocytes, or vice versa. This is illustrated by Case 55, which also shows how difficult it can be to interpret the serological results, and the multiple serological difficulties which can arise in the same case.

CASE 54

Mrs. G.Q. was referred to us for investigation following a clinical diagnosis of acquired haemolytic anaemia and the finding of a positive direct Coombs test. The serological results were as follows:

ABO group

Patient's cells			*Patient's serum*		
Anti-A	*Anti-B*	*AB serum*	*A cells*	*B cells*	*O cells*
v	—	—	—	v	—

Conclusion: Group A.

Rh group

Saline agglutinins:	anti-C	+++
	anti-D	v
	anti-D	v
	anti-E	—
Saline control	.. ..	—
Incomplete antibody (serum albumin) anti-c		v
AB serum/serum albumin control		v

Conclusion: The Rh group is R_1R_1 or R_1r. The result with the anti-c is unsatisfactory, as the control shows. Unfortunately no saline agglutinating anti-c of sufficient strength was available.

BLOOD GROUPING DIFFICULTIES IN CASES OF ACQUIRED HAEMOLYTIC ANAEMIA

In this chapter it is proposed to describe difficulties of a serological nature that arise in cases of auto-immune acquired haemolytic anaemia. In these cases there is evidence of the occurrence of auto-antibodies. It is not proposed to deal with clinical or other features of these cases, but only with the difficulties that arise in respect of blood grouping work or the provision of compatible blood for transfusion.

Dacie (1954) classifies auto-immune types of haemolytic anaemia as follows:

(*a*) Idiopathic acquired haemolytic anaemia.
(*b*) Haemolytic anaemia following virus pneumonia and certain other infections.
(*c*) Paroxysmal cold haemomoglobinuria.
(*d*) Haemolytic anaemia associated with chronic lymphatic leukaemia, reticulo-sarcoma, or disseminated lupus erythematosus, etc.

We shall not deal with Lederer's anaemia nor with cold paroxysmal haemoglobinuria nor the Donath-Landsteiner reaction. For details, readers are referred to Dacie (1954). Thus, the other cases fall into two classes, the idiopathic type in which the aetiology is unknown, and those secondary to virus pneumonia, or secondary to some malignant or other disease.

These cases may be subdivided into two groups (Dacie, 1954):

1. The warm antibody type.
2. The cold antibody type.

Cases also occur which appear to be intermediate in reaction.

Warm Antibody Type

In the warm antibody type of case, the direct Coombs test on the patient's cells is strongly positive with anti-γ-globulin reagent. Cases in remission may show similar results in the direct test. We have observed a case which has been in remission for a period of five years and still shows a strongly positive direct test. This may be the extent of the detectable serological change in the patient's blood; no antibodies may be detectable in the serum. In many cases, however, auto-antibodies are found in the serum of a non-specific, or specific, character. Weiner *et al.* (1953) have shown that these patients may possess specific auto-antibodies in their sera active against antigens present on their own red cells. These workers described a patient, of Rh group R_1R_1,

Representative Tests of Serum

Cells		Coombs test reagent		Serum albumin	Saline Agglutination	
No.	Group	Anti-γ	Anti-non-γ	37° C.	37° C.	16° C.
1	R_1R_1	++++	−	v	v	v
2	R_1r	++++	+	v	+++	v
3	R_1r	++++	−	v	+++	v
4	R_2R_2	++++	−	v	v	v
5	R_2r	++++	−	v	++	v
6	rr	+	−	++	−	v
7	rr	−	−	++	w	v
8	rr	−	−	++	−	v
9	rr	+	−	++	−	++
10	rr	+	−	++	−	−
11	rr	+	−	+++	−	+
12	R′	Agg.	Agg.	v	v	+++
13	R″	w	−	++	−	v
Own cells		+++	+++	+	w	+++

Tests of Eluate

These showed non-specific activity only and are therefore not given.

Conclusion

This case shows a number of interesting features.

1. The difficulty in determining the ABO group. (This could have been overcome by using Rh negative cells. See 6 below.)
2. The direct Coombs test was positive with anti-γ-globulin and anti-non-γ-globulin reagents, showing that this is a case intermediate in character between the cold and warm antibody types.
3. The serum showed anti-C+D specificity with the anti-γ-globulin reagent. This was probably due to the fact that she had been transfused with two bottles of group O Rh positive blood four years previously. The Rh group had been incorrectly determined at this time probably due to the autoagglutinin.
4. The Coombs tests with the R′ cells (No. 12) could not be performed owing to the presence of intense agglutination, suggesting that there was a saline agglutinating anti-C present, reacting as is usual more strongly with R′ than with R_1R_1 or R_1r cells.
5. The serum albumin tests showed the activity of the anti-C+D and a non-specific component.
6. The saline agglutination tests at 37° C. showed a specific anti-C+D agglutinin.
7. The saline agglutination tests at 16° C. showed a non-specific autoagglutinin. The weak reactions with cells No. 9, 10 and 11 appear to indicate that a specific antibody is present. This is misleading. These cells had been preserved in the frozen state whilst all the other cells were freshly collected. The weak reactions given by these three cells illustrate the labile character of the autoagglutinin receptor when cells are stored frozen.

Direct Coombs test

Anti-γ-globulin	+++++
Anti-non-γ-globulin ..	—
Saline	—

Representative Tests of Serum and Eluate

	Serum				Eluate			
	Coombs (anti-γ-globulin)	Serum albumin 37° C.	Saline 37° C.	Saline 16° C.	Coombs (anti-γ-globulin)	Serum albumin 37° C.	Saline 37° C.	Saline 16° C.
R_1R_1	+++	+++	—	—	++++	—	—	—
R_1r	++	+++	—	—	+++	+	—	—
R_2R_2	—	—	—	—	—	—	—	—
R_2r	+	w	—	—	—	—	—	—
rr	+	++	—	—	+++	w	—	—
rr	+	++	—	—	+++	+	—	—
rr	w	+	—	—	++	w	—	—

Conclusion: Both serum and eluate appear to have anti-C+e specificity. This was confirmed by further tests.

CASE 55

Mrs. E.W. was referred for serological investigation following the diagnosis of acquired haemolytic anaemia. The results were as follows:

ABO and Rh groups

The patient's cells taken directly from the specimen were spontaneously agglutinated, but following washing at 37° C. a free cell suspension was prepared.

	anti-A	anti-B	anti-C	anti-D	anti-E	anti-c	AB serum	A_1 cells	A_2 cells	B cells	O cells
16° C.	w	w	NT	NT	NT	NT	w	v	v	v	v
37° C.	—	—	—	—	—	v	—	v	v	v	+++

NT = not tested.

Conclusion: Group O, rr. The anti-A and anti-B content of the serum could not be determined since the serum reacted with group O cells even at 37° C.

Direct Coombs test

Anti-γ-globulin ..	+++
Anti-non-γ-globulin ..	+++
Saline	w

and 37° C. In the indirect Coombs test (anti-γ-globulin) the following results were obtained:

	Standard Cells										
	R_1r	R_1r	R_1R_1	R_1r	R_1R_1	rr	rr	rr	R_0r	R_2r	R_2R_2
Serum	+++	+++	+++	+++	+++	w	–	+	–	–	–
Eluate	++	+++	++	++	+++	w	–	+	–	–	–

Further tests showed that the antibody concerned had a predominantly anti-C specificity, but it also reacted weakly with certain rr bloods and was thought t

The Rh group could not

this time owing to the pos

sample of the patient's blood, examined a year later, was undoubtedly R_2r. In particular it gave negative results with nine different anti-C sera. The direct Coombs test was now negative and no antibody could be detected in the patient's serum.

Cold Antibody Type

These are much less frequent than the previous type and the direct antiglobulin test on the cells is positive provided that anti-non-γ-globulin reagent is used. Very weak reactions, only, occur in some cases with anti-γ-globulin reagent. The serum of the case usually contains high titre cold agglutinins. The titre and avidity of the agglutinin is less at higher temperature than low temperature. Cases do occur in which the autoagglutinins are apparently active against the red cells at 37° C. using the saline agglutination test. This is due to the manner of collection of the specimen and the method of examination employed, since once the autoantibody has seized hold of the red cells it is very difficult to dislodge it simply by raising the temperature of the agglutinates to 37° C. Gardner (1949) has drawn attention to the value of using acidified serum for the detection of antibodies in these cases. Dacie (1953) states that, using acidified serum, a weakly positive indirect antiglobulin test can be obtained using cells sensitized at 37° C.

Serological Tests

Difficulties arise in blood grouping in these cases because of the positive direct antiglobulin test on the patient's cells, or because of the presence of autoagglutinins which adhere to the red cells. Secondly, difficulties arise in providing compatible blood because of the presence of atypical antibodies in the patient's serum, some of which may be directed against antigens present on the patient's own red cells.

Tests of Patient's Cells

The cells should be removed from the sample of citrated blood, washed three times at room temperature and examined on a slide to see if the red cells are agglutinated. In many cases they will be found

Where a specific antibody is found in the serum which is not present in the eluate, it may be that it is an autoantibody associated with the haemolytic process, or that it is an antibody resulting from the antigenic stimulus of previous transfusions. This distinction is of no importance to the management of the case, since blood lacking the corresponding antigen must, in any case, be given. The distinction depends on whether the cells of the patient bear the corresponding antigen, though this may be difficult to determine if the antigen is masked by blocking.

CASE 56

The cells of Miss I.W. group $A_1R_1R_2$ were only weakly agglutinable by anti-c and anti-E sera, in spite of the fact that an eluate made from the patient's cells had an anti-c+E specificity. The c and E antigens could, however, be demonstrated after her cells had been *washed five times in saline at 43° C. A sample of blood taken fifteen* months later reacted normally with anti-c and anti-E sera although the direct Coombs test was still positive.

The presence of transfused cells in the patient's circulation may sometimes lead one to think that he does possess the antigen when, in fact, the antigen is only present on the transfused cells. If a specific antibody is found in the eluate, it is worth while to give blood lacking the corresponding antigen, even though this specific antibody cannot be detected in the patient's serum. Here it may be presumed that the patient is producing this antibody but that it is all being taken up by his own cells; blood lacking the corresponding antigen may be less readily destroyed in the patient's circulation than would blood bearing the antigen.

The picture is further complicated by the occasional occurrence of cases in which specific antibodies can be eluted from the cells even though the cells do not bear the corresponding antigen.

CASE 57

The blood of Mrs. L.S. was referred to us when she was found to have a strongly positive direct Coombs test associated with cirrhosis of the liver. Her haemoglobin level at this time was 13 gm. per cent, so th [illegible] quiescent. She had had three [illegible] ear previously. The serological [illegible]

Group OR_2r

Direct Coombs test

Anti-γ-globulin	.. ..	+++
Anti-non-γ-globulin	..	−

Both serum and eluate gave negative results with Group O standard cells in the serum albumin test and saline agglutination tests at 16° C.,

tests should be carried out by the following methods: Coombs test, serum albumin and saline agglutination at 37° C. and saline agglutination at 16° C. Tests with papainized cells may also be done if nothing is found by the other methods, but if all the tests by the other methods are strongly positive it is unlikely that the papainized cells will be helpful.

If any specificity is apparent, which may only be shown by differences in the strength of reaction and may not be entirely clear cut, further tests with suitable cells should be carried out to identify the antibody. Titrations of serum or eluate are sometimes useful here.

If no specificity is found in the tests of the serum and eluate, a specific antibody may well be present, nevertheless, but may be masked by a strong non-specific component. This problem would have to be tackled by absorbing the serum and eluate with each of the bloods mentioned above, and retesting the absorbed serum and eluate with all the bloods by all the methods. This, however, is a laborious procedure and very seldom, in our experience, does it show anything of interest. Often in these cases, if serum or eluate is absorbed with -D-/-D- cells, the absorbed serum or eluate will be found to give a negative result with -D-/-D- cells, but positive results with cells of other Rh groups. This is an interesting observation, the significance of which is not at present understood.

If a specific antibody is found in the serum or the eluate, blood lacking the corresponding antigen should be given. If no specificity is found and the serum gives positive results with all the standard cells it may be considered that all blood is incompatible, including the patient's own. If transfusion is essential, one can test a number of bottles and give those which seem to give the weakest reactions with the patient's serum, though it is doubtful whether this procedure is of much value.

COLD ANTIBODY TYPE. (Direct Coombs test predominantly or entirely due to anti-non-γ-globulin, often associated with a strong autoagglutinin, sometimes so strong that the Coombs test cannot be satisfactorily performed.)

There are three difficulties in these cases; firstly, if a specific antibody is present it is likely not to be an Rh antibody; secondly, complement is often necessary for the action of the antibody, and thirdly, the antibody itself tends to be thermo-labile.

Tests of the *serum* should be the same as for the warm antibody type, except that the Coombs test should be carried out using anti-γ-globulin and anti-non-γ-globulin, and the Coombs tests should be done at 16° C. as well as at 37° C.

Two *eluates* should be made; one by eluting the cells at 37° C. and removing the supernatant, and the second by taking these eluted cells and subjecting them to a second elution at 56° C. The temperature

to form a suspension free from agglutination. In certain cases they will be found to be agglutinated and if the slide is warmed at 37° C. this will disperse. These red cells should be washed with warm saline at 37° C. and re-examined until agglutination has dispersed and a free suspension of red cells remains at room temperature. In certain cases it will be found that the red cells will still be agglutinated after washing at 37° C. due to the presence of high titre autoagglutinin in the serum. The red cells should be then further washed with saline at 42° C. and re-examined. Washing with saline should not be at a temperature higher than 44° C. or the erythrocyte antigens may be damaged. In the majority of cases this will succeed in providing a free suspension of red cells, which should be tested as follows:

1. ABO AND RH GROUP. The cells should be tested with anti-A, anti-B and AB serum. The patient's serum should be tested with A cells, B cells and O cells, but if the serum contains non-specific or other antibodies, this part of the test may give unsatisfactory results. The Rh group should be determined using anti-C, anti-D, anti-E, anti-c, anti-e (if available) and AB serum.

Sera acting as saline agglutinins should be used and high titre anti-A, anti-B and anti-Rh sera diluted in saline are preferable to low titre sera used undiluted.

Careful ABO and Rh grouping is essential in these cases because false results occur frequently and the cross-matching test may be so difficult and unsatisfactory as to be virtually useless. Occasionally, some of the Rh antigens are blocked by specific auto-antibodies and in these cases the antigen may be revealed after the antibody has been eluted from the red cells. This is a relatively hazardous procedure, however, since it must be remembered that the Rh antigen is damaged by a temperature of 56° C. (Murray and Clark, 1952).

2. DIRECT COOMBS TEST. Anti-γ-globulin and anti-non-γ-globulin should be used together with a saline control. This test will indicate whether the case is likely to be of the warm or cold antibody type.

INVESTIGATION OF SERUM AND ELUATE

WARM ANTIBODY TYPE. (Positive direct Coombs test predominantly or entirely due to anti-γ-globulin; little or no autoagglutination.) Test the serum and eluate with R_1R_1, R_2R_2, and three rr bloods bearing as many blood group antigens as can be managed. The three rr bloods should not only bear as many of the blood group antigens as possible, but should also be such that a blood lacking each of these antigens is present. If all were S positive, for example, one might mistake an anti-S for a non-specific antibody. Cells of the same ABO group as the patient should also be included. The

CHAPTER XII

THE IDENTIFICATION OF ATYPICAL ANTIBODIES IN HUMAN SERA

In this chapter the recommended methods are those which will enable known antibodies to be identified. It is not intended to suggest how new antibodies can be identified, although by using these methods they may be, nor is it intended to suggest how any new antibodies might be linked or associated with an existing blood group system. The statistics in Chapter XIII are similarly concerned with the identification of known antibodies.

Atypical antibodies are defined as all blood group antibodies other than anti-A and anti-B. Such antibodies may arise in susceptible individuals as a result of blood transfusion, or other parenteral injection of blood, or following pregnancy.

If the stimulus is blood transfusion, almost any antibody can occur in the serum depending on individual susceptibility. For example, it seems probable that the Kell antigen may be as antigenic as Rh, but fewer anti-Kell antibodies are found because the stimulus is less frequently given. Since homologous blood with respect to the D antigen is customarily transfused, atypical antibodies following blood transfusion do not nowadays usually include anti-D. Where pregnancy is the cause of the iso-immunization, the commonest antibodies are those produced by the Rh antigens, anti-D, anti-C+D, anti-E, anti-c, anti-D+E, together with anti-K, in that order of frequency. These antibodies are often accompanied by others, such as anti-Le^a, anti-Le^b, anti-P and anti-M.

Antibodies may arise without obvious iso-immunization. These are anti-H, anti-O, anti-P, anti-Le^a, anti-Le^b, anti-M, anti-N, anti-Wr^a, anti-Mi^a, anti-M^g, and occasionally the Rh antibody anti-E. Many of these antibodies are active only at temperatures lower than 37° C. although occasionally they are active at this temperature also, and are commonly so in the case of Lewis antibodies.

In Chapter I it was pointed out that whereas antibodies may be considered to be of natural occurrence, antigens similar to those present on human red cells may be widely distributed in Nature. Heterogenetic immunization may thus result in the production of antibodies in human sera which are active against antigens present on red cells.

Cameron and Staveley (1957) described the occurrence of blood group P substance in hydatid cyst fluids. They showed that strong anti-P agglutinins might be present in the sera of these persons and considered that they were a selected group presenting a high proportion

should not exceed these figures. In order to add complement, two volumes of eluate should be mixed with one volume of fresh serum, lacking A, B, Le^a and Le^b group specific substances. Such a serum may be stored at −70° C. *Tests of the eluate* are the same as the tests of the serum. Controls of the standard cells with the fresh serum by all the methods will also be needed. Following the results of these tests, proceed as described for the warm antibody type.

Each case presents its own problems so that the methods to be used have to be adapted to the individual case and no set scheme will cover all eventualities. It is hoped, however, that these observations will be helpful in assisting those who endeavour to provide blood for transfusion for these difficult cases. The main emphasis is laid, firstly, on getting the ABO and Rh group of the patient correct, and secondly, on detecting any specific antibodies which may be present in the red cell eluate or in the serum, or both. This is done by the investigation detailed above, and by drawing upon one's experience of what antibodies are known to occur most frequently, and making specific tests. It is often possible to provide blood which one feels is relatively satisfactory for the patient but sometimes it is impossible to provide apparently compatible blood.

References

Callender, Sheila T., and Race, R. R. (1946). A serological and genetical study of multiple antibodies in response to blood transfusion by a patient with lupus erythematosus diffusus. *Ann. Eugen.*, 13, 102.

Dacie, J. V. (1953). Acquired haemolytic anaemia with special reference to the antiglobulin (Coombs) reaction. *Blood*, 8, 813.

Dacie, J. V. (1954). *The haemolytic anaemias. Congenital and acquired.* London: J. and A. Churchill Ltd.

Gardner, F. H. (1949). Transfer to normal red cells of an agglutinin demonstrable in the acidified sera of patients with acquired haemolytic jaundice. *J. clin. Invest.*, 29, 814.

Murray, J., and Clark, E. C. (1952). Production of anti-Rh in guinea pigs from human erythrocyte suspensions. *Nature*, 169, 886.

Weiner, W., Battey, D., Cleghorn, T. E., Marson, F. G. W., and Megnell, M. J. (1953). Serological findings in a case of haemolytic anaemia. *Brit. Med. J.*, ii, 125.

the antibody were determined using papainized red cells. The results of this test are summarized in Table 63.

TABLE 63. ILLUSTRATING RESULTS IN CASE 59

Cells	*Papainized cell technique, 16° C.*
R_1R_1	++++
R_1R_1	+++
R_1r	++++
R_1r	+++
R_2r	w
R_2r	w
R_2R_2	++++
R_2R_2	+++
R′r	++++
R″r	—
R_0r	+++
R_yr	++++
rr	—
rr	—
rr	—

A distinction must be made between the detection of antibodies, and their identification, which is the determination of their specificity. Antibodies need to be identified following their detection in laboratory procedures; for example, in cross-matching tests or antenatal tests, or because of discrepancies arising during ABO typing. Alternatively, tests for detection may be similar to those required for preliminary identification of the antibody and under certain circumstances, where it is thought that atypical antibodies might be present in a serum, these two procedures are combined and carried out at the same time. This occurs when sera are examined:

1. Following reactions to blood transfusion in a patient.
2. From mothers who have given birth to children affected with haemolytic disease of the newborn (or where this is suspected). and where no antibodies were detected antenatally.
3. From patients who have received multiple blood transfusions.
4. From cases of acquired haemolytic anaemia.

Materials

1. PANEL OF STANDARD CELLS

(a) READILY AVAILABLE. The panel of standard cells consists of a number of group O cells which have been grouped as fully as possible. In the standard scheme set out on p. 253 five cells are used as preliminary test cells and subsequently another batch of two or three

of unusually strong anti-P sera. They found that hydatid cyst fluid contained P substance when live scolices were present but not when they were absent.

Rh antibodies are occasionally present in sera in the absence of obvious iso-immunization. In this regard, careful questioning of the patient or donor will sometimes reveal a history of having received intramuscular injections of blood previously. Nevertheless, cases do occur in which there is no such history, and in which Rh antibodies are present in the serum. Naturally occurring Rh antibodies are rare but the commonest of these is anti-E, which may be accompanied by anti-Wr^a. Recently when searching for anti-Wr^a a naturally occurring anti-E antibody was detected. We have never seen anti-D as a naturally occurring antibody in serum, but an unusual Rh antibody was observed in the serum of a male donor and was apparently unrelated to iso-immunization.

CASE 58

J.M., aged 77 years, suffering from chronic urinary obstruction due to benign hypertrophy of the prostate. He had never been transfused and, so far as he knew, had never been injected with blood. He was group O Rh negative. Anti-E antibody was present in his serum active at room temperature only against papainized red cells. The results are shown in Table 62.

TABLE 62. TESTS ON SERUM FROM CASE 58

Cells	*Papainized slide technique*		*Antiglobulin test*	*20% Albumin test*	*Saline agglutination*	
	20° C.	*37° C.*			*20° C.*	*37° C.*
R_1R_1 .	–	–	–	–	–	–
R_2R_2 ..	++++	–	–	–	–	–
R_2r ..	+++	–	–	–	–	–
R_2r .	+++	–	–	–	–	–
R_1r	–	–	–	–	–	–
rr .	–	–	–	–	–	–
rr .	–	–	–	–	–	–
rr .	–	–	–	–	–	–
Own cells	–	–	–	–	–	–

CASE 59

A male blood donor, aged 33, had never been transfused or injected with whole blood. The antibody was first detected during routine screening for antibodies. He was group O, R″r and an unusual Rh antibody was present in his serum. The antibody was not detected using the antiglobulin test or the albumin agglutination test. The saline agglutination test was negative and the reactions of

The Basis of Identification

This rests upon:

1. Tests of a preliminary nature with a panel of standard cells.
2. Confirmatory tests.
   (*a*) Further tests with standard cells, if necessary.
   (*b*) Demonstration of the absence of the antigen or antigens on the person's red cells against which the antibody present in the serum is directed; this does not apply in cases of acquired haemolytic anaemia.
   (*c*) Qualitative and quantitative examination of the characteristic reactions of the antibody.

The statistical considerations on which the identification is made are given in Chapter XIII. It may be said, briefly, that the considerations which have led to these recommendations arise from the fact that the full groups of the standard test cells are, in the majority of cases, known and the diagnosis is based on identification by exclusion.

Many antibodies have characteristic reactions. If an antibody of a certain specificity appears to be present in a serum but does not have the characteristic reactions possessed by the majority of such antibodies, then one should very carefully reconsider whether the specificity has been correctly determined, e.g. Case 71, p. 267. It may well be that it has, and indeed antibodies are known in some cases to give unusual reactions.

Scheme of Identification

PRELIMINARY TEST

Standard preliminary test. The serum is tested with five blood samples, all group O, the full groups of which are detailed in Table 64. It will be observed that two of them are Rh positive, R_1R_1 and R_2R_2, and three are Rh negative. These cells are selected so as to vary as much as possible with respect to their other blood groups. R_1R_1 and R_2R_2 blood groups are particularly selected as the Rh positive test cells since they are most useful for the identification of Rh antibodies. The three Rh negative cells are selected so that they contain all other commoner blood group antigens between them. They are also selected so that each one contains one of the relatively rarer antigens, for example, one is Le(a+), a second is Lu(a+) and a third K+. Cells of these

TABLE 64. CELL SAMPLES USED IN PRELIMINARY TEST

Sample:									
N.	.. O, R_1R_1	M	Ss	P+	Lu(a−)	K−	Le(a−b+)	Fy(a−)	Jk(a+)
JB.	.. O, R_2R_2	M	Ss	P+	Lu(a−)	K−	Le(a−b+)	Fy(a+)	Jk(a+)
S.	.. O, rr	N	ss	P+	Lu(a−)	K−	Le(a+b−)	Fy(a−)	Jk(a+)
C.	.. O, rr	MN	ss	P+	Lu(a+)	K−	Le(a−b+)	Fy(a+)	Jk(a+)
H.	.. O, rr	M	Ss	P+	Lu(a−)	K+	Le(a−b+)	Fy(a+)	Jk(a−)

times this number may be required for complete identification. The five standard cells should be tested with anti-D (complete with tests for D^u), anti-C, anti-E, anti-c, anti-e, anti-M, anti-N, anti-S, anti-P, anti-Lu^a, anti-K, anti-Le^a, anti-Le^b, anti-Fy^a, anti-Jk^a. For the complete investigation of Rh antibodies, cells of type R'r, and R"r, and cells tested with anti-C^w, anti-C^x and, in some parts of the world, anti-V, are useful. If possible, it is desirable in special laboratories that a number of suitable cells should also be tested with anti-s, anti-Jk^b, anti-Lu^b and anti-Fy^b to distinguish homozygotes from heterozygotes. It is an advantage if many of the test cells are Rh-negative, since if the serum contains anti-D as well as other antibodies, this will make identification of these easier.

Many antibodies show dosage effects; they have higher titres or higher avidities when tested with the homozygote, than when tested with the heterozygote. When searching for antibodies which show this dosage effect it is an advantage if the test cells are of homozygous type. It is not always possible to select such cells, especially in the preliminary test, but they are always included wherever possible.

(b) FROZEN CELLS AS OCCASIONAL SOURCES OF REFERENCE. It is often possible to obtain, usually with help from one's colleagues, samples of red cells of rare blood groups which should be kept frozen solid for special use. These will include group OKK, group OC^w positive, group OC^x positive, and similar cell samples of rare groups.

2. STANDARD ANTISERA

Standard antisera are also required because one of the most important confirmatory tests is the blood grouping of the person's cells in whose serum the antibody was found. Standard antisera generally required in the average blood grouping laboratory are:

Anti-A	Anti-M
Anti-B	Anti-N
Anti-A_1	Anti-S
Anti-H	Anti-P
Anti-D	Anti-Lu^a
Incomplete anti-D for detecting D^u	Anti-K
Anti-E	Anti-Le^a
Anti-C	Anti-Le^b
Anti-e	Anti-Fy^a
Anti-c	Anti-Jk^a

Many additional sera, of course, are valuable if they are available.

The number of standard red cells and the extent to which they are tested, and the number of standard antisera available in a laboratory, will determine the extent to which antibodies can be identified. Those listed in these sections will be mainly confined to blood grouping laboratories.

TABLE 65. THEORETICAL RESULTS GIVEN BY PRELIMINARY TEST

Cells	Results with:														
	Anti-D	Anti-C	Anti-E	Anti-c	Anti-e	Anti-K	Anti-Le^a	Anti-H	Anti-P	Anti-M	Anti-Le^b	Anti-S	Anti-Lu^a	Anti-Fy^a	Anti-Jk^a
N.	+	+	−	−	+	−	−	+	+	+	+	+	−	−	+
JB.	+	−	+	+	−	−	−	+	+	+	+	+	−	+	+
S.	−	−	−	+	+	−	+	+	+	−	−	−	−	−	+
C.	−	−	−	+	+	−	−	+	+	+	+	−	+	+	+
H	−	−	−	+	+	+	−	+	+	+	+	+	−	+	−

particular groups may not be available regularly; for example, sometimes cells of group R_1r or R_2r have to be substituted for those shown in the table.

The techniques used are as follows:

1. Saline agglutination tests in tubes at 16° C. and 37° C.
2. Serum albumin tests in tubes at 37° C.; preferably performed in the form of a titration.
3. Antiglobulin tests using anti-γ-globulin reagent and anti-non-γ-globulin reagent.
4. Papainized cell tests, if appropriate, following 1, 2 and 3 above.

The patient's own cells should also be tested by all these means with his own serum.

Theoretical results which are given by the common antibodies using these five test cells are shown in Table 65.

Because of dosage effects (e.g. anti-c) and the occurrence of weak and strong reactors (anti-P) the theoretical results may not always be obtained. Nevertheless, a number of distinctive results may be found even at this stage. Using these five test cells certain antibodies give the same reactions, but even under these circumstances a clue might be given as to which of the antibodies it is because of characteristic reactions of the one or the other. For example, in distinguishing anti-Le^b from anti-M, haemolytic activity of the former might be helpful. The results of this preliminary test can be summarized as follows:

ANTIBODY PRESENT AND IDENTITY INDICATED. If it is thought that an antibody has been identified, it must be tested with three or more red cell samples that lack this particular antigen but contain all other common antigens, and three red cell samples that possess the antigen (see Chapter XIII for details). In most cases in which the identity of the antibody is indicated and in which the results of the tests are characteristic of the majority of similar antibodies, the only additional confirmatory test that need be done is to ensure that the red cells of the patient in whom this antibody was found lack the antigen. For example, if the antibody is found to be anti-D, giving characteristic reactions and coming from an Rh negative patient who has received an Rh positive stimulus, there is no more that need be done.

CASE 60

Mrs. R. received a blood transfusion for haematemesis and since 1932 has had ten pregnancies. She then gave birth to a full-term stillborn child and her serum was tested and found to contain an atypical antibody (Table 66). The results with standard cells indicate the identity of the antibody as anti-c. This antibody gave the charac-

TABLE 68. FURTHER TESTS IN CASE 61 USING PAPAINIZED RED CELLS

Cell sample	Results	
	Rh Group	*Papainized cell test*
O.	R_1R_1	—
D.	R_2r	+++
A.	R_2r	+++
V.	R_1r	—
Ba.	R_1^wr	w
C.	R_1R_2	—
B.	R_1R_2	—
H.	rr	+++

CASE 62

Mrs. S. received multiple blood transfusions and during the cross-matching test incompatibility was detected. Investigation of her serum using the standard cells gave the results shown in Table 69. Here the identity of the antibody was indicated as anti-K. The patient was group Okk, and the antibody gave the characteristic reactions of anti-K antisera. The bottle of blood with which the incompatibility was originally found was grouped and was group OK+. Further tests of this antibody with several cells, K+ and K−, containing all other common blood group antigens, confirmed its identity.

TABLE 69. TESTS ON SERUM FROM CASE 62

Cells	*Antiglobulin test*	*Serum albumin* 37° *C.*	*Saline agglutination*	
			16° *C.*	37° *C.*
N. R_1R_1 K− ..	—	—	—	—
JB. R_2R_2 K− ..	—	—	—	—
S. rr K− ..	—	—	—	—
C. rr K− ..	—	—	—	—
H. rr K+ ..	++++++	+++	—	+++
Own	—	—	—	—

CASE 63

Mr. B.T., admitted to hospital because of haematemesis, had received transfusions on many previous occasions. During the cross-matching test an incompatibility was found in the anti-globulin test only. The serum was referred for investi[illegible]

teristic reactions of an anti-c antibody. The patient was group AR_1R_1, and her husband group OR_1r. Further tests with cells of type R_1R_1, containing all other common blood group antigens, gave negative reactions and it was concluded that the identity of the antibody was established.

TABLE 66. TESTS ON SERUM FROM CASE 60

Cell	*Antiglobulin test*	*Serum albumin 37° C.*	*Saline agglutination*	
			16° C.	*37° C.*
N. R_1R_1	—	—	—	—
JB. R_2R_2	+++	+++++	+	++
S. rr	+++	++++	+	+
J. rr	+++	++++	+	+
C. rr	+++	++++	+	+
Own	—	—	—	—

CASE 61

Mrs. H. was delivered of a jaundiced infant suspected of being affected with haemolytic disease of the newborn and her blood was referred for investigation. [illegible] cells and the results are sl[illegible] not available on this parti[illegible] used instead of the homozygotes. Mrs. H. was group OR_1R_1, and her husband, group OR_2r. She had received no previous blood transfusions. The identity of the antibody was not indicated by these tests but it was thought possible that it might be an anti-c antibody showing dosage effects. Further tests were undertaken [illegible] in [illegible] led [illegible] to react with samples of blood of groups R_2r and R_1R_2. It is interesting to observe that it gives a weak reaction with blood of group $R_1^w r$.

TABLE 67. TESTS ON SERUM FROM CASE 61

Cells	*Antiglobulin test*	*Serum albumin 37° C.*	*Saline agglutination*	
			16° C.	*37° C.*
V. R_1r ..	—	—	—	—
D. R_2r	+++	—	—	—
S. rr .. .	+++	—	—	—
J. rr .. .	+++	—	—	—
C. rr	+++	—	—	—
Own	—	—	—	—

antibody reacted as a saline agglutinin at this temperature and gave such great differences between the strengths of the reactions suggested that it might be anti-P. The patient was P-negative. Further investigation with P-positive and P-negative cells confirmed the identity of the antibody as anti-P.

TABLE 71. TESTS ON SERUM FROM CASE 64

Cells	*Antiglobulin test*	*Serum albumin test 37° C.*	*Saline agglutination* 16° C.	37° C.
N. R_1R_1 P+ ..	–	–	++	–
JB. R_1R_1 P+ ..	–	–	+++++	–
S. rr P+ ..	–	–	+++	–
H. rr P+ ..	–	–	+++++	–
C. rr P+ ..	–	–	+++++	–
Own	–	–	–	–

ANTIBODY PRESENT, ALL THE TESTS NEGATIVE. It may seem curious that this can occur, but the reason for it should be sought in the reason why it was originally considered that an antibody was present in the serum at all. This may have been the result of a positive reaction between the serum and a single cell sample. If all the results are negative in the preliminary testing it is obvious that the standard red cells could not contain the antigen present in the red cell which was used when the antibody was originally detected. In other words, it suggests that the antibody is one active against a low frequency antigen. The procedure in a case where all tests are negative is illustrated by Case 69 (p. 264).

ANTIBODY PRESENT, ALL THE TESTS POSITIVE. In these circumstances it will be necessary to test the serum with a wider range of standard cells. It may be that two or more antibodies are present in the sera. Case 68 (p. 262) shows the procedure when all the tests are positive.

FURTHER TESTS AND CONSIDERATIONS

1. TESTS WITH A WIDER RANGE OF STANDARD CELLS. *If a probable identification of antibody has been made, the antibody gives characteristic reactions, and if the person's red cells in whose serum it appears lack the corresponding antigen, it will only need to be tested with three standard cells which are positive for the particular antigen and three which are negative for the antigen but contain all other common antigens. In the case of anti-C+D and anti-D+E, R′ and R″ test cells will need to be included as well. However, if no identification has been possible, a wider range of test cells will need to be employed. If the serum contains several antibodies, fifteen or more*

is anti-Fy^a. Tests showed that the patient was group OrrFy(a−). Nevertheless, during the course of testing this serum for antibodies it had been tested using papainized red cells and a positive result was found with Rh positive cells but not with Rh negative cells. Since it was known that anti-Fy^a was inactive using the papainized cell technique, further investigations were made. It was found that the serum contained an anti-Fy^a antibody active in the antiglobulin test and an anti-D antibody active against papainized red cells. It is interesting to note that the serum "PRI" described by Race, Sanger and Lehane (1953), which contained an anti-Fy^a capable of agglutinating cells suspended in saline, also had a weak anti-D antibody detectable only by the trypsin cell technique.

TABLE 70. TESTS ON SERUM FROM CASE 63

Cells	*Antiglobulin test*	*Serum albumin* 37° C.	*Saline agglutination* 16° C.	*Saline agglutination* 37° C.
N. O, R_1R_1 Fy(a−)	−	−	−	−
JB. O, R_2R_2 Fy(a+)	++	−	−	−
S. O, rr Fy(a−)	−	−	−	−
C. O, rr Fy(a+)	++	−	−	−
H. O, rr Fy(a+)	++	−	−	−
Own	−	−	−	−

ANTIBODY PRESENT AND IDENTITY NOT INDICATED. The results may fail to correspond with any known antigen, but nevertheless the qualitative nature of them might suggest what further tests should be performed. The results might be doubtful, or equivocal, or suggest the presence of more than one antibody, and it should be borne in mind that a mistake in technique, in the standardization of the original cells, or in recording, may produce results which are apparently confusing. If a weak antibody which shows a marked dosage effect is present, positive reactions will be given with homozygous cells, and possibly negative ones with heterozygous cells. This will depend upon the number of test cells present in the panel and sensitivity of the techniques employed.

Tests using a wider range of standard cells are required to make identification certain.

CASE 64

M.T., admitted to hospital with haematemesis and melaena. No previous blood transfusions. Four bottles of group ORh positive blood were put up for cross-matching and in the saline agglutination test at room temperature three of them were found to give positive results of variable strength. The serum was put up with the standard cells and the results are shown in Table 71. An antibody, active only as a saline agglutinin at 16° C., was detected. The fact that this

standard cells are shown in Table 73. He was found to be group O rrK-negative. Tests against a large range of standard cells indicated the presence of anti-E and anti-K. The antibody detected during the cross-matching test was anti-K, and the bottle of blood was group OrrK-positive. It seems likely that during his transfusions, he had received a bottle of blood of group R"r. The results of tests with a wider range of standard cells are shown in Table 74.

TABLE 74. SERUM OF CASE 66 TESTED AGAINST A WIDER RANGE OF STANDARD CELLS

Standard cell		*Antiglobulin test*
R_1r	K−	−
R_1R_1	K−	−
R_0r	K−	−
R_2r	K−	+++
R_1R_2	K−	++
R_2R_2	K−	++++
R_2r	K+	++++
R_1r	K+	+++
R_1r	K+	+++
rr	K+	+++
R'r	K−	−
R"r	K−	++++

CASE 67

Mr. W., suffering from Di Guglielmo's disease, had a reaction following a blood transfusion. His serum was referred [illegible]

groups were A_1B, rr, MNs, P+, K−, Le(a−b+), Fy(a−), Jk(a+).

TABLE 75. TESTS ON SERUM FROM CASE 67

Cells			*Antiglobulin test*	*Serum albumin* 37° C.	*Saline agglutination*	
					16° C.	37° C.
N.	O, R_1R_1	K−	−	−	+++	−
JB.	O, R_2R_2	K−	−	−	+++	−
S.	O, rr	K−	−	−	+++	−
C.	O, rr	K−	−	−	++++	−
H.	O, rr	K+	+++++	+++	++++	++++
Own	A_1B rr	K−	−	−	−	−

different red cells selected to be of value in this work are needed both for identification and to satisfy the statistical criteria. Such cells are those which are homozygous and heterozygous for as many blood groups as possible and known to include P-strong and P-weak types and cells with whose particular reactions the worker should be familiar.

CASE 65

The serum of Mrs. T. was examined during pregnancy and the results suggested the presence of an antibody. The results with the standard cells are shown in Table 72. Here the identity of the antibody was thought to be anti-Fy^a with the possibility of anti-Kell in addition, and tests with a further range of standard cells were indicated. The patient's group was OrrMNS P+, K−, Le(a−b−), Lu(a−), Fy(a−). Subsequent tests with a large range of standard cells showed the presence of anti-Kell and anti-Fy^a but no anti-D was detected.

TABLE 72. TESTS ON SERUM FROM CASE 65

Cells	*Antiglobulin test*	*Serum albumin 37° C.*	*Saline agglutination 16° C.*	*Saline agglutination 37° C.*
N. O, R_1R_1 Fy(a−) K−	−	−	−	−
JB. O, R_2R_2 Fy(a+) K−	+++	−	−	−
S. O, rr Fy(a−) K−	−	−	−	−
C. O, rr Fy(a+) K−	+++	−	−	−
H. O, rr Fy(a+) K+	++++	+++++	+	++
Own	−	−	−	−

CASE 66

Mr. K. suffered from aplastic anaemia and had received at least 60 pints of packed cells. During a cross-matching test, an incompatibility was detected and his serum was referred for investigation. He was group ORh negative, and so far as was known he had always received Rh neg. blood. Results of testing his serum with the

TABLE 73. TESTS ON SERUM FROM CASE 66

Cells	*Antiglobulin test*	*Serum albumin 37° C.*	*Saline agglutination 16° C.*	*Saline agglutination 37° C.*
N. O, R_1R_1 K−	−	−	−	−
JB. O, R_2R_2 K−	+++	+++++	−	−
S. O, rr K−	−	−	−	−
C. O, rr K−	−	−	−	−
H. O, rr K+	++++	+++++	−	−
Own	−	−	−	−

pregnancy her serum was examined routinely for the presence of antibodies and a positive result was obtained using the papainized cell slide technique. Her serum was put up with the standard cells and the results are shown in Table 77. The antibody reacted positively with all the red cells against which it was tested. The mother's blood was grouped and she was found to be O, rr, MNS, P+, Le(a−b−), K+, Lu(a−), Fy(a+). It was observed that the reaction with the anti-K serum was the strongest that had ever been seen using this serum. Her serum was put up with a further 15 standard cells, all of which reacted positively with it; it was obvious that her antibody reacted with a high-frequency antigen. The fact that her red cells gave such a strongly positive result with anti-K serum suggested that she was genotype KK and that the antibody in her serum might be anti-k. This was supported by the fact that it reacted less strongly with Kell-positive cells. The frozen cell panel of the laboratory contained two cells, genotype KK, one group O, R_1R_2, KK, [illegible] O, [illegible]
[illegible]
[illegible]
contain anti-D. This was in spite of the fact that the husband was group OR_1rMNS, Le(a−b+), kk, Lu(a−), Fy(a+).

TABLE 77. TESTS ON SERUM FROM CASE 68

Cells	*Antiglobulin test*	*Serum albumin test, 37° C.*	*Saline agglutination test* 16° C.	*Saline agglutination test* 37° C.	*Papainized slide test*
N. O, R_1R_1 K−	+++++	+++++	+	++	++++
JB. O, R_1R_2 K−	+++++	+++++	+	++	++++
S. O, rr K−	+++++	+++++	+	++	++++
C. O, rr K−	+++++	+++++	+	++	++++
H. O, rr K+	+++	+++	−	+	++
Own	−	−	−	−	−

The mother was subsequently delivered and the child affected with haemolytic disease of the newborn. Its red cells gave a strongly positive direct Coombs test and were group O, R_1r, Kk. An eluate was made from the cord cells, and the presence of anti-k was detected in this eluate, and in the cord serum, but no anti-D was present. This case illustrates the value of fully grouping the patient's erythrocytes, secondly, the value of the frozen cell panel, and thirdly, the value of elution of the cord cells.

Where doubtful antibodies are present in a serum it is advantageous to carry out the blood grouping of the patient's cells with more than one particular antiserum, if at all possible. This, again, may help to indicate the nature of the antibody present in the serum by drawing attention to peculiarities of the reactions between the various antisera and the red cells; Case 69 illustrates this.

Tests on a large number of standard cells showed that he had an anti-Kell antibody present in his serum giving positive results in the antiglobulin test. The results of further testing are shown in Table 76. The other antibody was considered to be anti-H active as a saline agglutinin at 16° C.

TABLE 76. FURTHER TESTS WITH STANDARD CELLS IN CASE 67

Cells				Saline agglutination	
				16° C.	37° C.
O,	R_1r	K−	..	+++	−
O,	R_2r	K−	..	+++	−
O,	R_0r	K−	..	+++	−
O,	rr	K−	..	+++	−
A_1,	R_1R_1	K−	..	−	−
A_1,	rr	K−	..	−	−
A_2,	R_1r	K−	..	+++	−
A_2,	R_2r	K−	.	+++	−
A_2,	rr	K−	..	+++	−
O,	rr	K+	.	+++	++++
O,	R_1r	K+	.	+++	+++
A_1B,	rr	K+	..	++	+++
A_1B,	rr	K−	..	−	−
A_1B,	rr	K−	..	−	−

2. **BLOOD GROUPING TESTS ON CELLS.** The red cells of the donor or patient in whose serum the atypical antibody is being identified should be grouped as completely as possible. Antibodies present in a person's serum are not normally active against antigens on their red cells. The antigenic analysis of the red cells, therefore, will give an indication of the nature of the antibody or antibodies which might be present in the serum (Cases 68, 69). This does not apply to cases of acquired haemolytic anaemia (Chapter XI). If a full range of blood grouping sera is not available and it is not possible to determine in every case whether the patient is homozygous or heterozygous for a particular antigen, then one should watch the results closely for possible dosage effect, bearing in mind those antibodies which might be liable to give rise to this, e.g. anti-c (Case 61, p. 256). If, when using a particular antiserum, it is found to give a very strongly positive result with a particular red cell sample it might suggest that the red cell is homozygous for that particular antigen. This, again, may be of help in the diagnosis of the antibody present in the serum.

CASE 68

Mrs. H. had two children and during her second pregnancy she received 2 pints of group ORh negative blood. During her third

5. Whether, in the antiglobulin test, a positive result is best obtained using anti-γ-globulin reagent or anti-non-γ-globulin reagent.
6. Whether decomplementation of the serum abolishes its activity in the antiglobulin test.
7. Whether group-specific substances or saliva are effective in neutralizing antibody activity.

Table 78 illustrates this.

CASE 70

Mrs. W., a 6th para, had been known to have anti-D antibodies in her serum for eight years. She was admitted to hospital for delivery and her serum was tested at the 35th week when the presence of anti-D antibody was confirmed; no other atypical antibodies were identified using the standard cells. She was group A, Rh negative, and her full groups were subsequently found to be A_2rrMNs P+, Lu(a−), kk, Le(a+b−), Fy(a+), Jk(a−). Her husband was group OR_1R_1, and the child's cord cells, group AR_1r, Jk(a+).

On admission to hospital she was delivered of a severely affected child, group A, Rh positive. The direct Coombs test was strongly positive using anti-γ-globulin reagent, but negative using anti-non-γ-globulin reagent. The cord haemoglobin was 12 gm. per cent, and it was decided to give an exchange transfusion. A cross-matching test was put up with several bottles of group A, Rh negative blood, using the mother's serum. The antiglobulin tests in all cases were positive, but the saline agglutination tests were negative.

Late at night the case was referred to us for investigation and was one of some urgency. The cross-matching test was repeated with several bottles of group A, Rh negative blood when it was found that the saline agglutination tests were negative, the serum albumin tests negative, but the antiglobulin test was positive using anti-non-γ-globulin reagent alone. It was decided to identify the antibody. Not all of the standard panel of cells were available that night, but quite a number were, together with some Rh positive ones. It was decided, therefore, to take advantage of the fact that the anti-D present in the serum using this reagent might not interfere with the tests. The results are shown in Table 79. The only Jk(a−) cell available was the cell Bsn. which was also R_1^ur. The serum was inactivated and the tests repeated. Compatible blood was provided that night and the antibody was confirmed as anti-Jk^a. The cord serum was found to contain anti-D antibody but not anti-Jk^a.

This case illustrates the following:

1. The fact that the activity of anti-D in this serum could be avoided by using anti-non-γ-globulin reagent (p. 61) enabling an R_1^ur cell to be employed.
2. The value of knowledge of the characteristic reactions of antibodies, which suggested anti-Jk^a originally (together with other possibilities), but which more strongly suggested that it was anti-Jk^a when the results disappeared on inactivation of the serum.

CASE 69

Mrs. X. was delivered of a full-term child affected with haemolytic disease of the newborn. The direct Coombs test was positive. The maternal serum was put up with the standard cells and the results were entirely negative throughout. The mother's cells were grouped and found to be $A_1R_1R_2$MNS, P+, Le(a−b+), K−, Lu(a+), Fy(a+), and the father, A_1R_1rMNS, P+, Le(a−b+), K−, Lu(a+), Fy(a+). The grouping of the father's red cells was undertaken with several anti-C antisera and different reactions were found; some reacted very weakly, and it was thought possible that there was some unusual kind of C antigen present in the father's erythrocytes. A joint test between the father's cells and the mother's serum was undertaken and a positive result was obtained in the saline agglutination test at 37° C. in the Coombs test, and particularly using the papainized cell technique. This latter method gave a much higher titre than using any of the other methods. This suggested that the antibody might belong to the Rh series of antibodies, and coupled with the abnormal reactions obtained when the father's cells were tested with anti-C sera suggested that it might be some antibody against the C antigen. Subsequent testing revealed this to be a new Rh antibody, anti-C^x (Stratton and Renton, 1954). This case illustrates the value of (1) careful testing of cells with the various antisera; (2) joint test in cases of haemolytic disease; (3) the help given by the study of the characteristic reactions of the antibody.

Blood grouping of the husband's blood in cases in which an atypical antibody is present during pregnancy may be looked upon as confirmation of the nature of the immunizing antigen, and may give a clue as to the nature of the antibody present in the maternal serum, as illustrated in the above case. It may, however, equally well show that the antibody present in the serum is not a result of pregnancy but probably a result of transfusion, or intramuscular injection of blood given previously. We have seen thirty-six cases in which a D negative mother married to a D negative husband had Rh antibodies present in the serum which were attributable, in our opinion, to previous injection of D positive blood.

3. CHARACTERISTIC REACTIONS OF THE ANTIBODY. Many antibodies have characteristic reactions and these are common to the majority of antibodies of that particular specificity, in many instances. The characteristics which should be considered are:

1. The technique which results in the strongest positive reaction between the standard cells and the serum.
2. The optimum temperature of activity.
3. Haemolytic activity of the antiserum.
4. Whether the agglutinates show any particular features in the saline agglutination test.

5. Whether, in the antiglobulin test, a positive result is best obtained using anti-γ-globulin reagent or anti-non-γ-globulin reagent.
6. Whether decomplementation of the serum abolishes its activity in the antiglobulin test.
7. Whether group-specific substances or saliva are effective in neutralizing antibody activity.

Table 78 illustrates this.

CASE 70

Mrs. W., a 6th para, had been known to have anti-D antibodies in her serum for eight years. She was admitted to hospital for delivery and her serum was tested at the 35th week when the presence of anti-D antibody was confirmed; no ot[illegible] identified using the standard cells. She [illegible] and her full groups were subsequently [illegible] Lu(a−), kk, Le(a+b−), Fy(a+), Jk(a−). Her husband was group OR_1R_1, and the child's cord cells, group AR_1r, Jk(a+).

On admission to hospital she was delivered of a severely affected child, group A, Rh positive. The direct Coombs test was strongly positive using anti-γ-globulin reagent, but negative using anti-non-γ-globulin reagent. The cord haemoglobin was 12 gm. per cent, and it was decided to give an exchange transfusion. A cross-matching test was put up with several bottles of group A, Rh negative blood, using the mother's serum. The antiglobulin tests in all cases were positive, but the saline agglutination tests were negative.

Late at night the case was referred to us for investigation and was one of some urgency. The cross-matching test was repeated with several bottles of group A, Rh negative blood when it was found that the saline agglutination tests were negative, the serum albumin tests negative, but the antiglobulin test was positive using anti-non-γ-globulin reagent alone. It was decided to identify the antibody. Not all of the standard panel of cells were available that night, but quite a number were, together with some Rh positive ones. It was decided, therefore, to take advantage of the fact that the anti-D present in the serum using this reagent might not interfere with the tests. The results are shown in Table 79. The only Jk(a−) cell available was the cell Bsn. which was also R_1^ur. The serum was inactivated and the tests repeated. Compatible blood was provided that night and the antibody was confirmed as anti-Jk^a. The cord serum was found to contain anti-D antibody but not anti-Jk^a.

This case illustrates the following:

1. The fact that the activity of anti-D in this serum could be avoided by using anti-non-γ-globulin reagent (p. 61) enabling an R_1^ur cell to be employed.
2. The value of knowledge of the characteristic reactions of antibodies, which suggested anti-Jk^a originally (together with other possibilities), but which more strongly suggested that it was anti-Jk^a when the results disappeared on inactivation of the serum.

TABLE 78. DISTINGUISHING CHARACTERISTICS OF THE COMMONER ANTIBODIES

Antibody	*Best technique*	*Papain*	*Haemolysins*	*Appearance of agglutination*	*Type of antiglobulin reagent*	*Comments*
Anti-Rh (all specificities)	Papain and Coombs test	Good enhancement	No	Normal	Anti-γ	Generally fails to react with saline cell suspensions on slides at room temperature.
Anti-M Anti-N	Saline, 16° C.	Inactive	No	Normal	Rarely positive	Incomplete antibodies very rare.
Anti-P	Papain and saline, 16° C.	Good enhancement	Rarely	Normal	Rarely positive	Incomplete antibodies very rare.
Anti-K Anti-k	Coombs or serum, 37° C.	No enhancement	No	Normal	Anti-γ	Better against cells suspended in human serum than against cells suspended in bovine albumin.
Anti-Lu^a	Saline, 16° C	No enhancement	No	May look like mixed agglutination	Rarely positive	Incomplete antibodies rare.
Anti-Le^a Anti-Le^b	Saline, 16° C.	Enhancement	Sometimes	Diagnostic cell chains	Mainly anti-non-γ	Mainly diagnosed by saline agglutination.
Anti-Fy^a	Coombs	Inactive	No	Normal	Either anti-γ or anti-non-γ	Acts usually by antiglobulin test.
Anti-Jk^a	Coombs	Inactive	No	Normal	Anti-non-γ	Acts usually by antiglobulin test.

TABLE 79. TESTS ON SERUM FROM CASE 70

Cells	Antiglobulin test			
	Fresh serum		*Inactivated serum*	
	Anti-γ-globulin	*Anti-non-γ globulin*	*Anti-γ-globulin*	*anti-non-γ globulin*
N. R_1R_1	+++++	+++++	+++++	−
JB. R_2R_2	+++++	+++++	+++++	−
S. rr	−	+++++	−	−
C. rr	−	+++++	−	−
Bsn. $R^{s}r$ Ns Jk(a−)	++++	−	++++	−
Own	−	−	−	−

3. The importance of cross-matching blood with the maternal serum, since although this antibody did not in this case appear to [illegible] far as [illegible]
4. [illegible] which in our previous experience had shown some very weak positive results with certain cell samples, led us to reduce the proportion of cells to serum in the antiglobulin test and this enabled strong positive results to be obtained in the cross-matching test.

We were surprised at the high percentage of positive results that were obtained when tests were made on Rh negative cells using this serum. It seems that this antibody reacted strongly under these conditions of the test with a number of weakly reactive Jk(a+) cell samples.

Sometimes several antibodies are present in the same serum and under these circumstances not only the preliminary test with the standard cells but the characteristic features of the antibodies, the groups of the donors, and very many of the things previously referred to in this chapter, all go to help in the separation and identification of these antibodies. This is illustrated by Case 71.

CASE 71

Mrs. L.B., group A_1R_0, never had a transfusion but had two normal children followed by three jaundiced children and one miscarriage. Haemolytic disease was suspected and preliminary tests showed that an antibody was present. The first step toward its identification was to do the preliminary test (Table 80).

The reactions with cell samples JB and D suggested anti-E and that with sample C anti-Lu^a. The serum was therefore tested with a further series of specially selected bloods (Table 81).

TABLE 80. TESTS ON SERUM FROM CASE 71

Cells	*Antiglobulin test*	*Serum albumin 37° C.*	*Saline agglutination* 37° C.	16° C.
N. O, R_1R_1	—	—	—	—
JB. O, R_2R_2 ..	+++	++	—	+
S. O, rr	—	—	—	++
C. O, rr	++	++	++	+
H. O, rr	—	—	—	++
W. O, R′	—	—	—	+
D. O, R″	++	++	w	+
Own	—	—	—	—

TABLE 81. FURTHER TESTS ON THE SERUM OF CASE 71

Cell sample No.	*Groups*	*Anti-globulin test*	*Serum albumin 37° C.*	*Saline agglutination* 37° C.	16° C.
8	O, rr Lu(a−)	—	—	—	+
9	O, R_1R_1 Lu(a−)	—	—	—	++
10	O, R_1r Lu(a+)	++	+	—	+
11	O, R_2r Lu(a−)	+++	++	—	++
12	O, R_2r Lu(a−)	+++	++	—	+
Own cells		—	—	—	—

These further tests confirmed the suggestion of anti-E plus anti-Lu^a, though a third antibody active only at 16° C. was also present. The antigenic constitution of the test samples excluded anti-C, D, c, e, f, M, N, S, s, P, K, k, Le^a, Le^b, Lu^b, Fy^a, Fy^b, Jk^a and Jk^b active at 37° C.

The full groups of Mr. and Mrs. L.B. were now determined. These were:

Husband: A_1, R_2r, MNS, P+, Le(a+b−), K−, Lu(a+), Fy(a+), Jk(a+), Wr(a−)
Wife: A_1, R_0, NS, P+, Le(a−b−), K−, Lu(a−), Fy(a+), Jk(a+), Wr(a−).

The reaction of the husband's cells with anti-E was onl[illegible] d *further tests showed that his cells were* [illegible] portion of anti-E sera. He [illegible] The rather [illegible] nature of [illegible] that furthe[illegible] Her serum [illegible] of selected cell samples by [illegible] albumin tests at 37° C. as follows:

Groups	*Number of bloods tested*	*Result with maternal serum*
O, E+, Lu(a+)	1	Positive
O, E+, Lu(a−)	6	All positive
O, E−, Lu(a−)	5	All negative
O, E−, Lu(a+)	6	All positive

It was thus reasonably certain that the antibodies were anti-E and anti-Lu^a. We were now able to turn our attention to the third antibody active only at 16° C. This antibody reacted with P-negative cells, with M-negative, and with N-negative, and with Le(a−b−) cells, and anti-O or anti-H was therefore suspected. It was tested at 16° C. as follows:

Groups	*Number of bloods tested*	*Result*
O, E−, Lu(a−)	4	All positive
A_1, E−, Lu(a−)	7	All negative
A_2, E−, Lu(a−)	1	Positive

Further tests showed that the antibody was not inhibited by secretor saliva, and it was therefore considered to be anti-O. This serum thus contains anti-E, anti-Lu^a and anti-O.

Special Cases

1. Antibodies to Low-frequency Antigens

It may be found during a laboratory procedure that one cell sample reacts with the patient's serum giving a positive result, whereas all the other red cell samples fail to do so. Furthermore, it will be found that when the serum is put through the standard scheme, and even possibly tested against further standard cells, the results are all negative (Case 69). This suggests that the serum, if it contains an antibody, contains one against a rare antigen.

Under such circumstances it should first be established that the positive result is due to the presence of an atypical antibody in the patient's serum and does not indicate the occurrence of some other type of antigen-antibody reaction. Three alternative causes of antigen-antibody reactions are:

1. that the cell possesses a rare ABO antigen, for example, A_4, B_2, not routinely recognized as such;
2. that there are changes in the sample resulting in it being poly-agglutinable;

3. that the reaction is due to the false positive results described in Chapter V.

Firstly, therefore, one should exclude these possibilities, and a fresh sterile sample should be obtained. The cell sample should be tested with a number of group O sera known to detect rare sub-groups of group A or B, as described in Chapter VI. Polyagglutinability should be excluded by testing the cells with 20–50 fresh normal sera at 16° C. using the saline agglutination tube technique. Polyagglutinability cannot be distinguished from bacteriogenic effects, excepting where it is known that the sample is sterile. Tests should show that the direct antiglobulin test on the cell is negative, using anti-γ and anti-non-γ-globulin reagents. Remember, here, to make sure that the cells are not coated with cold incomplete anti-H antibody.

The serum should be tested with cell samples containing low-frequency antigens until the identity of the antibody is established. It may help to group the cell sample originally found to give positive reactions with the serum under examination, for rare antigens.

2. Antibodies to High-frequency Antigens

In these circumstances (Case 68) all the tests obtained with a large panel of standard cells are likely to be positive. This may be due to an auto-antibody, and certain auto-antibodies, especially from cases of acquired haemolytic anaemia, appear to be active at 37° C. Anti-H or anti-O will need to be excluded, p. 123, since such sera may occasionally react at a higher temperature with all group O red cells irrespective of their other blood groups, although in the majority of cases these antibodies are active only at low temperature (Case 71). The person's own red cells should also be negative with their own serum using all tests. High-frequency antibodies are shown in Table 117 (p. 313).

Anti-Le^a and anti-Le^b when present together in the same serum, and both strongly active, should be included in this series, since such sera may react positively with more than 90 per cent of standard group O test cells. They may even react positively with certain red cells of type Le(a−b−), especially from Lewis secretor individuals. In detecting such a combination of anti-Lewis antibodies it is helpful to note that:

1. The patient's red cells are of type Le(a−b−).
2. Cells which give negative results with the antiserum are all of type Le(a−b−).
3. In the saline agglutination test the agglutinates show diagnostic features (p. 295).
4. Haemolytic activity may be observed. We have seen a case in which anti-Le^a and anti-Le^b were found together following a transfusion reaction where both antibodies acted as haemolysins against normal red cells.

References

Cameron, G. L., and Staveley, J. M. (1957). Blood group P substance in hydatid cyst fluids. *Nature*, 179, 147.
Race, R. R., Sanger, Ruth, and Lehane, D. (1953). Quantitative aspects of the blood-group antigen, Fy^a. *Ann. Eugen.*, 17, 255.
Stratton, F., and Renton, P. H. (1954). Haemolytic disease of the newborn caused by a new Rh antibody, anti-C^x. *Brit. Med., J.* i, 962.

STATISTICAL CONSIDERATIONS IN THE IDENTIFICATION OF ANTIBODIES

THE following method, though unconventional, is one which we have found satisfactory in practice. Its basis is that one should first test the unknown antibody in such a way as to exclude as many as possible of the other antibodies which it might be. Statistical considerations should only arise when this has been done and should only be used as a means of excluding those antibodies which it has not been possible to test for directly. The method is called:

Identification by Exclusion

The use of this method presupposes that the antibody gives clear-cut positive and negative results, agreeing in specificity with a certain antigen, and that all the serological results by which it is identified are correct. (The second postulate will be further considered below.)

The method is a confirmation of identity rather than a method of identification, and consists in showing that the antibody fulfils two criteria.

(i) Three positive results and three negative results with cell samples agreeing with its supposed specificity, e.g. three D positives and three D negatives if the antibody is thought to be anti-D.

Two positive and two negative results are sufficient if the antibody appears to be one of the commonest ones (anti-A_1, D, P), if it shows the characteristic mode of reaction expected from its specificity, and if it comes from a patient whose cells lack the corresponding antigen.

(ii) Negative results with cells bearing as many of the other known blood group antigens as can be managed. These must include C, D, E, c, e, f, M, N, S, s, P, Lu^a, K, Le^a, Le^b, Fy^a, Fy^b, Jk^a, Jk^b, and preferably C^w also.

The antibody is then considered to be satisfactorily identified.

The bloods used in these tests are not random blood samples but bloods selected for their antigenic constitution, and bloods may need

provide the requisite number of negative results in (i) as are used in (ii), so that in an ideal case only three bloods giving negative results might be needed to satisfy both criteria. Moreover, the results obtained

in the initial tests made to identify the antibody may themselves alone be sufficient if they satisfy the criteria. In other cases it is only necessary to carry out such additional tests as will satisfy the criteria when combined with the results of the initial tests.

A certain amount of difficulty is sometimes encountered in obtaining cell samples which are fully typed, and which are negative with the antibody under consideration. For example, in the case of anti-c, R_1R_1 cells fully typed for the other antigens would have to be used. Difficult though this may be at times, it is, nevertheless, essential before it can be said that the antibody has been satisfactorily identified. A practical example of this was an anti-k which we detected in the serum of a D negative antenatal patient. It was essential here to test the serum at least with D positive KK cells, in order to exclude the concomitant presence of anti-D. Lack of care in the selection of suitable standard cells may occasionally lead to difficulties when using this method. For example, we were, on one occasion, much perturbed to find that an anti-D serum had been identified by its positive results with two D positive Kell positive cell samples, and had not been tested with D negative Kell positive cells. The danger here is obvious, and is simply due to carelessness.

In using this, or any other statistical method, one has to be careful of three things. The first is that statistical methods can only show how closely two things are correlated. They can never show that they are identical. No amount of statistics will show, for example, whether an anti-C serum is really anti-C or anti-C+C^x, and an attempt to discover whether an anti-c serum also contains anti-E by means of statistical calculations would be extremely laborious if, indeed, it could be done at all. A single test, using C^x positive cells in the first case and R_1R_2 cells in the second, would give the answer at once. The second thing that has to be remembered is that "in blood group work we usually do not set out to compare anything, but make the comparisons *after* the work is done; the results may then be compared in many ways. The number of ways in which the work has been compared makes a big difference to the level of probability at which an observed association is considered to be 'significant' " (Race and Sanger, 1954). This means that, strictly speaking, one ought to first find out what the antibody is, and then repeat all the tests using a different series of cells, so that only a single comparison is being made. Since this would involve doing everything twice it is not often practised, but the second series of tests ought to be carried out if the antibody appears to be a rare one, if it shows unusual characteristics or if the statistical calculations do not give a low probability that the supposed identification is incorrect.

Thirdly, when a statistical test is applied repeatedly there is a cumulative chance that one will be misled sooner or later, and in laboratories where large numbers of sera are dealt with this may be quite important.

The value of this method of identifying antibodies by a process of exclusion may be illustrated by the fact that in the past four years we have detected and identified exactly a thousand new anti-D antibodies (with or without anti-C and anti-E) in the sera of antenatal patients. The identity of the majority of these has been confirmed by tests on subsequent samples of the patient's sera and by the birth of D positive children suffering from haemolytic disease. In no case has the diagnosis of anti-D been subsequently shown wrong, even though it was made by the use of only two or three D positive and three D negative cell samples in the first place.

Rationale of the Method

We now turn to the rationale of the method, and to the reasons why these two criteria are sufficient to give a satisfactory identification of the antibody.

If an antibody reacts with three D positive bloods and fails to react with three D negative bloods, it is likely that the antibody is anti-D. The question to which we seek the answer is: "What is the probability that it is not really anti-D but something else?" If it were possible to test the antibody against a series of D negative bloods which between them contained every known blood group antigen, and if all these tests were negative and we were sure that all the serological results were absolutely reliable, we would be able to conclude for certain that the antibody was either anti-D or a new and hitherto unknown antibody. Furthermore, it could only be a new antibody if, by chance, all the D positive bloods bore the corresponding antigen and all the D negative bloods lacked it. In practice, of course, we fall short of this ideal state of affairs, firstly because no serological results can be considered absolutely reliable, and secondly because bloods tested with every known blood group antibody are not available.

The question of the possible unreliability of the serological results either in testing the antibody which we are identifying, or in the initial grouping tests of the standard blood samples, can scarcely be subjected to statistical treatment. All that one can do to avoid being misled by this is to maintain a high standard of serological technique in the laboratory, and to view unexpected or bizarre results with suspicion and as requiring further confirmation. It is also wise when the antibody being identified appears to be of an unusual specificity, to avoid if possible relying at any stage in the reasoning on a single positive or negative result. Strict application of this rule on all occasions would, however, involve an almost complete duplication of the work which would scarcely be practicable.

The second way in which we fall short of the ideal is that bloods tested with every known blood group antibody are not available, and it is here that statistical considerations arise.

The frequencies of the blood group antigens are set out in Table 117 (p. 313) and it ought to be possible, in a laboratory equipped to undertake the identification of blood group antibodies, to obtain a selection of cells such that all those antibodies whose antigen frequencies lie between 99 per cent and 2·5 per cent can be excluded by direct tests. (It may be noted that it is not necessary to have standard cells which have been tested with anti-c, anti-s, anti-Fy^b or anti-Jk^b, since cells which are negative with anti-E, anti-S, anti-Fy^a and anti-Jk^a respectively can be presumed to be positive with the first four sera.)

The antibody which we are investigating gives the reactions +++ −−− with the three pairs of cell samples, agreeing with its supposed specificity mentioned in Criterion 1, and we can be misled only if the antibody is not, in fact, anti-Q (to take a purely hypothetical example), but some other antibody which by chance gives the same reactions with the three pairs of cells. The chance of this occurring depends on the frequencies of the antigens and on the frequencies of occurrence of the antibodies. The latter are only very approximately known (Table 115, p. 312).

The probability that the antibody is not anti-Q can be calculated as follows:

Let f_Q be the relative frequency with which anti-Q occurs; and
Let f_x, f_y, f_z be the relative frequencies with which the antibodies anti-X, anti-Y, anti-Z, etc., which we have not been able to exclude, occur.

Since they are relative frequencies.

$$f_Q+f_x+f_y+f_z+\ldots=1$$

Let p_x, p_y, p_z, etc., be the probabilities that the bloods tested will have that antigenic constitution which could mislead us in the case of each antibody.

Consider, for example, anti-Kp^a. p_{Kp^a} is the probability that the three Q positive bloods would all be Kp(a+) and the three Q negative bloods would all be Kp(a−). Since the frequency of Kp(a+) bloods is 2 per cent,

$$p_{Kp^a}=(0{\cdot}02\times0{\cdot}98)^3$$
$$=7{\cdot}5\times10^{-6}$$

Now, of the antibodies with which we are here concerned (which comprise anti-Q and all the antibodies which could not be excluded):

The proportion which are anti-Q is f_Q, and all these react +++ −−− with the six bloods.
The proportion which are anti-X is f_x, and $f_x\,p_x$ react +++ −−− with the six bloods.
The proportion which are anti-Y is f_y, and $f_y\,p_y$ react +++ −−− with the six bloods.

And so on.

The total of those which react ++++ −−−−, but which are not anti-Q, is:

$$f_x\,p_x+f_y\,p_y+f_z\,p_z+\ldots$$

Therefore, of all the antibodies which react ++++ −−−, the proportion which are not anti-Q is:

$$\frac{f_x\,p_x+f_y\,p_y+f_z\,p_z+\ldots}{f_Q+f_x\,p_x+f_y\,p_y+f_z\,p_z+\ldots}$$

and this is the same as the probability $P_{not\,Q}$ that the antibody is not anti-Q.

Now,

$$f_x+f_y+f_z+\ldots=1-f_Q$$

and if p_x is the greatest of p_x, p_y, p_z, etc., it follows that

$$f_x\,p_x+f_y\,p_y+f_z\,p_z+\ldots$$

is less than

$$p_x\,(f_x+f_y+f_z+\ldots)$$

which equals

$$p_x\,(1-f_Q)$$

So that

$$P_{not\,Q}<\frac{p_x\,(1-f_Q)}{f_Q+p_x\,(1-f_Q)}$$

Since $1-f_Q$ is less than 1

$$P_{not\,Q}<\frac{p_x}{f_Q+p_x}$$

This expression is true for all values of p_x and f_Q, but can be made more useful if the actual values with which we are dealing are taken into account.

Now p_x can be calculated from the frequency of the commonest antigen* with which the serum has not been tested. This is Kp^a, frequency 2 per cent, so that, as seen above,

$$p_x=p_{Kp^a}=7\,5\times10^{-4}$$

Since p_x is so small, we assume that it is negligible compared with f_Q, and write

$$P_{not\,Q}<\frac{p_x}{f_Q}\text{ or }\frac{7{\cdot}5\times10^{-4}}{f_Q}$$

The value of $P_{not\,Q}$ now only depends on f_Q, and would only be large if f_Q were very small.

If the hypothetical anti-Q is one of the commonest antibodies, f_Q will not be less than 0·1, so that $P_{not\,Q}$ will be $<7{\cdot}5\times10^{-5}$ or 1/13,300; and even if f_Q is as small as 0·001, as might happen with a rare antibody, $P_{not\,Q}$ will still be $< 7{\cdot}5\times10^{-3}$ or 1/133.

These are the probabilities of error using three pairs of positive and negative results in Criterion (i).

The use of only two pairs of positive and negative results increases p_x to 0 0004, so that with the commonest antibodies where f_Q might be as small as 0·1, $P_{not\,Q}$ will be $<1/250$, and the identification will still be satisfactory. With rarer antibodies, however, where f_Q might be as low as 0·001, $P_{not\,Q}$ can be as high as 0·4, which is obviously unsatisfactory.

The conclusion to be drawn from these considerations is that three pairs of positive and negative results can be considered to give a satisfactory identification of the antibody unless it appears to be an antibody of a very exceptional character. Two pairs of positive and negative results can only be considered satisfactory in the special case where the antibody appears to be one of the commonest ones (anti-A_1, D, P), where it shows the characteristic mode of reaction expected from its specificity, and where it is known to come from a patient whose cells lack the corresponding antigen.

Reference

Race, R. R., and Sanger, Ruth (1954). *Blood Groups in Man.* 2nd Ed. Oxford: Blackwell Scientific Publications.

CHAPTER XIV

BLOOD GROUP SYSTEMS OTHER THAN ABO AND RH

In this chapter various blood group systems will be described, including the antigens of the red cells and the corresponding antibodies.

Some of the antigens of these systems are of common occurrence whilst others are very rare. Likewise, some of the antibodies occur relatively frequently in sera and others are only very exceptionally present. The antibodies of the different systems tend to have their own characteristic mode of reaction in the different tests, and a knowledge of this is often of great value in identifying the antibody and in preparing and using the various grouping sera. However, it must not be forgotten that antibodies of the same specificity do not always follow the set pattern, so that an antibody showing features not characteristic of antibodies of that specificity may be encountered. Antibodies may show a thermal optimum different from that usually attributed to antibodies of the same specificity, or may be enhanced by serum albumin, or active in the antiglobulin test on occasion, though this is not the usual pattern of antibodies of that particular specificity. Some antibodies act best or entirely with anti-γ-globulin reagents and some with anti-non-γ-globulin reagents, but here again exceptions to the usual pattern are seen.

Blood grouping sera for the purpose of carrying out tests for these special blood groups are difficult to procure in many instances, because of their rarity or because of the difficulty of finding those which are sufficiently potent. In many instances one has to use such sera as are available and the tests have to be carried out by whatever technique gives the best results with the particular antibody concerned.

Blood grouping sera may be of animal or human origin. Those of animal origin are mainly concerned with MN typing alone, and others are obtained from the sera of normal individuals or those who have become immunized.

These human sera should be examined as described in Chapter XII, to determine whether any blood group antibodies, other than the one which is required, are present. Tests against cells bearing as many of the blood group antigens as possible must always be included, employing the technique by which it is proposed to use the serum, though similar tests by other techniques should be carried out also. If such a serum is to be used as a blood grouping serum all antibodies other than the particular one required must be absorbed out. Anti-A and anti-B will have to be removed in order to make the serum universally applicable, and other antibodies such as anti-D may have to be removed also in order to make the serum specific. Sera containing only a single

antibody are preferable to sera containing multiple antibodies, since where multiple antibodies are present the unwanted ones may be difficult to remove by absorption and the repeated absorptions which may be needed may weaken the antibody which it is desired to retain. Attempts to separate antibodies of the same system, e.g. anti-Le^a+Le^b, are often unsuccessful. A potent and specific serum from a group AB person is a great prize, since it requires no absorption. Such sera as anti-P and anti-Le^a should be sought in group AB individuals.

Some sera may be inactivated by heating at 56° C. for 25 minutes before absorption, but some examples of typing sera, such as anti-Jk^a and incomplete anti-Le^a and anti-Le^b should not be inactivated, since their activity depends on complement or other fresh serum factors. Absorption will, therefore, need to be carried out to remove anti-A and anti-B using the procedure described on p. 54.

Where antibodies to high-frequency antigens, such as anti-k, are to be absorbed to make them specific and universally applicable, it may not be possible to find cells, for example, of genotype KK, and yet of the appropriate ABO group. Under these circumstances the best that can be done is to add group specific substances to the serum. Such addition, however, may not entirely eliminate incomplete anti-A which, in itself, may give a positive antiglobulin test. It may be necessary, therefore, to make a careful study of the nature of the anti-A or anti-B present in the serum to ascertain whether the addition of saliva, or group specific substances, will produce a reliable typing serum which can be used for all ABO groups.

Animal sera require absorption to remove species specific antibodies and often to remove antibodies to A and B cells also. A mixture of groups A, B, and O cells of other suitable blood group combinations should be used.

Blood grouping sera are stored as previously described. The only point which need be re-emphasized here is that absorbed sera may, although specific when originally absorbed, lose this specificity and antibodies return to the serum for unknown reasons. Constant testing is necessary to ensure that specificity is maintained.

Controls are very necessary in all blood grouping work. When using the sera described in this chapter they are absolutely essential. This is because the sera are often difficult to obtain so that weakly active sera may have to be used, because the sera are used less frequently than ABO and Rh grouping sera and so may have become inactive or non-specific, and because the sera may give unfamiliar types of agglutination or may have to be used by unfamiliar techniques.

Two types of control are needed; a positive control to show that the antibody present is acting, and one or more negative controls to show that absorption is satisfactory and that non-specific activity is absent. The cells used as the positive control should be cells with the weakest

antigen likely to be encountered in the tests. These will be heterozygous for the antigen concerned and will be weak reactors in those cases where variations of antigenic strength occur. The correct positive control cells are indicated in each section. The correct negative controls depend on the serum to be used and sometimes on the cells to be tested also. It is essential to control the absorption by using, as a negative control, cells with antigens corresponding to the antibodies which have been removed, and this control also serves to show that the serum is not giving positive results with all cells (e.g. if it has become infected). With some antibodies it is difficult to secure control cells with the required combination of groups, and if it is known that the cells to be tested do not possess the antigen corresponding to the antibody which has been removed from the serum, then the absorption control will be unnecessary, but a negative control to guard against non-specific activity must still be included.

Many of the causes of false positive and false negative results discussed in Chapters IV and V apply to the determination of the groups described in this chapter, and certain additional causes of difficulty peculiar to the individual systems are discussed in the appropriate sections.

Different red cell samples often give different titres or scores with the same test serum. This is often associated with the fact that the samples are homozygous or heterozygous for that particular antigen although differences may occur within each group. Some sera are specially able to show this dosage difference between one red cell and another using a particular technique, and dosage is more readily demonstrable in some blood group systems than in others.

The MNS Blood Group System

The MN groups were originally discovered by Landsteiner and Levine (1927a and b), and the S subdivision following the discovery of anti-S in a serum in 1947 by Walsh and Montgomery. Subsequently anti-s was described by Levine, Kumichel, Wigod and Koch in 1951. This made complete genotyping with respect to these groups possible. Table 82 shows the MN groups and their genotypes and frequencies:

TABLE 82. MN GROUPS

Groups	*Genotypes*	*Approx. frequency (percentage)*
MN	MN	50
M	MM	28
N	NN	22

and Table 83 the MNSs groups and their frequencies:

TABLE 83. MNSs GROUPS AND THEIR FREQUENCIES
(frequencies after Race and Sanger, 1954)

MNS group	*M*		*N*		*MN*	
MNSs genotypes	MSMS MSMs	MsMs	NSNS NSNs	NsNs	MSNS MSNs MsNS	MsNs
Frequency ..	20%	8%	7%	15%	28%	22%

Table 83 is based on the use of anti-M, anti-N and anti-S antisera. Anti-s antiserum is very rare and therefore the full frequencies of all groups are not given.

Some further rare antigens belonging to the MNS system are known. M_2 (Jakobowicz *et al.*, 1949), N_2 (Andresen, 1947) and M^c (Dunsford *et al.*, 1953) are weak forms of the M and N antigens. Hunter (Landsteiner *et al.*, 1934) and Henshaw (Chalmers *et al.*, 1953) are two antigens which are common in Negroes but rare in Caucasians. They have specific antibodies—anti-Hunter and anti-Henshaw.

In the case of Miltenberger (Mi^a) (Levine *et al.*, 1951) and Verwyst (Vw) (van der Hart *et al.*, 1954) the antigens are rare but the antibodies are not uncommon (Wallace *et al.*, 1957.) Vw positive bloods seem to be Mi(a+) also, but the reverse is not the case. Both these antigens were originally thought to be "family" antigens and each came to light during the investigation of a case of haemolytic disease of the newborn, where it was found that the mother's serum reacted with the cells of the father but not with any of a large number of random bloods. Later it was shown that both antigens belong to the MNS system and that the two are closely connected, and further examples of Vw positive and Mi(a+) cells were found. Anti-Mi^a and anti-Vw were found to occur quite frequently in sera and appear to be naturally occurring. The situation resembles that found with the rare antigen, Wr^a (p. 304).

The antibody anti-S^u (anti-U), also belongs to this system (Wiener *et al.*, 1953; Greenwalt *et al.*, 1954). S^u-negative individuals are only found amongst Negroes and their cells give negative results with both anti-S and anti-s.

It is obvious that the MNS system is one of the more complicated blood group systems, and that we are a long way from a complete understanding of it.

ANTI-M, ANTI-N AND ANTI-S IN HUMAN SERA

Anti-M occurs rarely, and anti-N very rarely, in human sera. They are active as saline agglutinins at 16° C. and sometimes at 37° C. We have observed eight examples of anti-M. Two of these were in associa-

tion with anti-D and six occurred alone in the serum. Four were detected during antenatal testing, two during blood grouping and occurred in normal healthy blood donors, and two during the performance of the cross-matching test. All the persons in whose sera these antibodies occurred were blood group N. All reacted as saline agglutinins at 16° C. and two still gave positive results at 37° C. Sera would occasionally give positive results at 37° C. using the serum albumin technique when the result in the saline agglutination technique was negative. The serum albumin technique, therefore, should always be employed when these antibodies are sought. Dosage effects may be demonstrated (Table 84).

TABLE 84. DOSAGE EFFECT DEMONSTRATED BY HUMAN ANTI-M SERUM (WA) TITRATED IN SALINE AT 16° C.

Cells	*Titre*									*Score*
	1	2	4	8	16	32	64	128	*Control*	
H. O M	+++++	++++	+++	++	++	w	–	–	–	16
FS. O MN	++	++	+	+	w	–	–	–	–	6
Ha. O N	–	–	–	–	–	–	–	–	–	0

CASE 72

Mrs. M., aged 31 years, had three normal full-term deliveries and no miscarriages. The last child was said to have been jaundiced at birth. The mother received a blood transfusion of whole blood in 1937 with good clinical effect and no reaction. She was group A_1 rr, NS, and her husband was group A_1, rr, MS. The maternal serum contained anti-D and anti-M, the former resulting, presumably, from the blood transfusion. In the fourth pregnancy labour was induced at 38 weeks and the child was jaundiced for the first 24 hours. The cord haemoglobin at birth was 14·5 gm. per cent. The cord red cells were A, rr, MNS. The direct Coombs test was negative. The Wassermann reaction and Kahn test on both parents were negative. The maternal serum reacted strongly with the child's cord cells at 37° C. The cord cells showed no spherocytosis or abnormal fragility. Anti-M was present in the cord serum. When the child was 1 month old it was readmitted to hospital with anaemia. Haemoglobin: 8·5 gm. per cent; RBC: 3,400,000; WBC: 10,200; Reticulocytes: 3 per cent. The child was given three simple blood transfusions of group A, rr, N blood, and made a complete recovery. The maternal serum titrated with M pos. cells had a titre

TABLE 85. TITRATION OF SERUM IN CASE 72

Cell samples	Technique		Titre								
			1	2	4	8	16	32	64	128	Control
O, rr, M	Saline agglutination	16° C.	+++	++	–	–	–	–	–	–	–
		37° C.	–	–	–	–	–	–	–	–	–
O, rr, MN		16° C.	++	–	–	–	–	–	–	–	–
		37° C.	–	–	–	–	–	–	–	–	–
O, rr, N		16° C.	–	–	–	–	–	–	–	–	–
		37° C.	–	–	–	–	–	–	–	–	–
O, rr, M	Serum albumin test	16° C.	+++++	+++++	+++	+++	++	+	–	–	–
		37° C.	++	w	–	–	–	–	–	–	–
O, rr, MN		16° C.	+++++	+++++	+++	++	w	–	–	–	–
		37° C.	–	–	–	–	–	–	–	–	–
O, rr, N		16° C.	–	–	–	–	–	–	–	–	–
		37° C.	–	–	–	–	–	–	–	–	–

One example of anti-N has been observed by us in a healthy blood donor which caused discrepant results in the ABO grouping. The agglutinin was active only in saline at room temperature. It was inactive at 37° C. The antiglobulin test at 37° C. was also negative.

Anti-S sera have been seen by us on two occasions. The first was following multiple blood transfusions, when it occurred in combination with anti-c antibody. The second occurred in a case of acquired haemolytic anaemia. In this latter case it may have been due to the associated blood transfusion, and the anti-S was associated with anti-Wr^a and anti-Mi^a. The antibody observed in the first case was active at 37° C. and detectable using the antiglobulin test alone (anti-γ-globulin reagent). In the second case the antibody was active as a saline agglutinin at room temperature but not at 37° C., although at the latter temperature a positive antiglobulin test was obtained using anti-γ-globulin reagent. There was a difference in the strength of reaction of the serum with heterozygous and homozygous S-positive cells (Table 86). This serum is used as a typing serum using the saline agglutination test at room temperature. Control tests of Ss and ss cells are always included. Owing to the dosage effect and the fact that certain heterozygous cells may give quite weak reactions with this serum, care has to be taken in the actual reading of the test.

TABLE 86. DOSAGE EFFECT DEMONSTRATED USING ANTI-S(HE)
The anti-s serum used to group the test cells was kindly supplied by Dr. E. Giblett.

Cell sample	Saline agglutination at 16° C.						Score
	1	2	4	8	16	Control	
O, M, SS ..	+++++	+	w	–	–	–	6
O, M, Ss ..	+++	w	–	–	–	–	3
O, M, Ss ..	++++	w	–	–	–	–	4

tion with anti-D and six occurred alone in the serum. Four were detected during antenatal testing, two during blood grouping and occurred in normal healthy blood donors, and two during the performance of the cross-matching test. All the persons in whose sera these antibodies occurred were blood group N. All reacted as saline agglutinins at 16° C. and two still gave positive results at 37° C. Sera would occasionally give positive results at 37° C. using the serum albumin technique when the result in the saline agglutination technique was negative. The serum albumin technique, therefore, should always be employed when these antibodies are sought. Dosage effects may be demonstrated (Table 84).

TABLE 84. DOSAGE EFFECT DEMONSTRATED BY HUMAN ANTI-M SERUM (WA) TITRATED IN SALINE AT 16° C.

Cells	*Titre*									*Score*
	1	2	4	8	*16*	32	64	*128*	*Control*	
H. O M	+++++	++++	+++	++	++	w	–	–	–	16
FS. O MN	++	++	+	+	w	–	–	–	–	6
Ha. O N	–	–	–	–	–	–	–	–	–	0

CASE 72

Mrs. M., aged 31 years, had three normal full-term deliveries and no miscarriages. The last child was said to have been jaundiced at birth. The mother received a blood transfusion of whole blood in 1937 with good clinical effect and no reaction. She was group A_1 rr, NS, and her husband was group A_1, rr, MS. The maternal serum contained anti-D and anti-M, the former resulting, presumably, from the blood transfusion. In the fourth pregnancy labour was induced at 38 weeks and the child was jaundiced for the first 24 hours. The cord haemoglobin at birth was 14·5 gm. per cent. The cord red cells were A, rr, MNS. The direct Coombs test was negative. The Wassermann reaction and Kahn test on both parents were negative. The maternal serum reacted strongly with the child's cord cells at 37° C. The cord cells showed no spherocytosis or abnormal fragility. Anti-M was present in the cord serum. When the child was 1 month old it was readmitted to hospital with anaemia. Haemoglobin. 8·5 gm. per cent; RBC: 3,400,000; WBC: 10,200; Reticulocytes: 3 per cent. The child was given three simple blood transfusions of group A, rr, N blood, and made a complete recovery. The maternal serum titrated with M pos. cells had a titre of 16 in saline at 16° C. and 2 at 37° C. The antiglobulin test was negative and the serum retested using anti-non-γ-globulin reagent has been found negative. Her serum at the present time reacts as in Table 85. Note the saline agglutination test negative at 37° C. but serum albumin test positive. The role of anti-M in this case was undecided.

TABLE 85. TITRATION OF SERUM IN CASE 72

Cell samples	Technique		Titre 1	2	4	8	16	32	64	128	Control
O, rr, M	Saline aggluti- nation	16° C.	+++	++	–	–	–	–	–	–	–
		37° C.	–	–	–	–	–	–	–	–	–
O, rr, MN		16° C.	++	–	–	–	–	–	–	–	–
		37° C.	–	–	–	–	–	–	–	–	–
O, rr, N		16° C.	–	–	–	–	–	–	–	–	–
		37° C.	–	–	–	–	–	–	–	–	–
O, rr, M	Serum albumin test	16° C.	+++++	+++++	+++	+++	++	+	–	–	–
		37° C.	++	w	–	–	–	–	–	–	–
O, rr, MN		16° C.	+++++	+++++	+++	++	w	–	–	–	–
		37° C.	–	–	–	–	–	–	–	–	–
O, rr, N		16° C.	–	–	–	–	–	–	–	–	–
		37° C	–	–	–	–	–	–	–	–	–

One example of anti-N has been observed by us in a healthy blood donor which caused discrepant results in the ABO grouping. The agglutinin was active only in saline at room temperature. It was inactive at 37° C. The antiglobulin test at 37° C. was also negative.

Anti-S sera have been seen by us on two occasions. The first was following multiple blood transfusions, when it occurred in combination with anti-c antibody. The second occurred in a case of acquired haemolytic anaemia. In this latter case it may have been due to the associated blood transfusion, and the anti-S was associated with anti-Wr^a and anti-Mi^a. The antibody observed in the first case was active at 37° C. and detectable using the antiglobulin test alone (anti-γ-globulin reagent). In the second case the antibody was active as a saline agglutinin at room temperature but not at 37° C., although at the latter temperature a positive antiglobulin test was obtained using anti-γ-globulin reagent. There was a difference in the strength of reaction of the serum with heterozygous and homozygous S-positive cells (Table 86). This serum is used as a typing serum using the saline agglutination test at room temperature. Control tests of Ss and ss cells are always included. Owing to the dosage effect and the fact that certain heterozygous cells may give quite weak reactions with this serum, care has to be taken in the actual reading of the test.

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Cell sample	Saline agglutination at 16° C. 1	2	4	8	16	Control	Score
O, M, SS ..	+++++	+	w	–	–	–	6
O, M, Ss ..	+++	w	–	–	–	–	3
O, M, Ss ..	++++	w	–	–	–	–	4

Preparation of Anti-M and Anti-N Grouping Sera

It is customary for routine work to use anti-M and anti-N sera prepared in rabbits, although human anti-M sera can be used on occasion.

The technique described below refers to the preparation of anti-M. The preparation of anti-N is, of course, similar.

Method of Immunization. Various methods have been described for the injection of rabbits to produce immune sera. For success it is necessary to inject a number of rabbits and select those which produce a high titre serum. Rabbits should be injected with 0·1 ml. of washed packed group OM cells intravenously on five successive days and rested for a period of two or three weeks, the course being repeated. It may be necessary to repeat the course of injections several times. The crude serum is titrated with cells of groups OM, ON and OMN, and it is essential that a titre of 1 in 2-3,000 against homologous cells should be obtained at the outset if a satisfactory reagent is to be produced after absorption (less for anti-N).

Absorption. The serum is inactivated at 56° C. for 25 minutes and diluted 1 in 10 to 1 in 40 according to the original non-specific titre of the serum. The absorbing cells should be of types AN, BN, and ON. The serum is absorbed initially according to the rapid method described on p. 25. Tests to ensure that a specific reagent has been prepared should be undertaken using the tube technique at room temperature. It should show a specific activity with M and MN cells and should be inactive with AN, BN and ON cells. The titre of the absorbed anti-M should be about 32 against homozygous cells. In the case of anti-N the titre should be about 8 against homozygous cells. Animals tend to respond more satisfactorily to injections of OM than of ON blood.

Technique of MN Grouping

A tube saline agglutination technique at room temperature is used, though with good sera a slide test may be used. Three anti-M and three anti-N typing sera prepared in different animals are used to test each cell sample. The cells are prepared from citrated blood and are washed three times in saline. One volume of each of the typing sera and one volume of the cell suspensions are put in the tubes and they are allowed to sediment at room temperature for one and a half hours. The tubes are gently tapped and the contents of those giving negative results are removed from the tube and examined under the low power of the microscope.

Positive and negative controls should be put up with each lot of tests. MN is the correct positive control for both anti-M and anti-N sera.

It is often recommended that in MN typing it is desirable from time to time to make a statistical check of one's results (Taylor *et al.*, 1939). This should be based on a minimum of 300 tests and an endeavour made to see whether the actual results obtained fit those expected. The χ^2 test can be used for this purpose. Statistical results are, of course, no substitute for serological accuracy.

FALSE RESULTS IN MN GROUPING

In MN grouping false results can be due to many causes (Chapter V). There are, however, two causes of false results which call for special mention. These are false positive results due to the presence of serum in cell suspensions, and secondly false negative or false positive results due to loss of specificity of the reagents.

Incompletely washed cells may, under certain circumstances, be falsely agglutinated by anti-M or anti-N typing serum. This is due to the fact that sub-agglutinating doses of rabbit-anti-human-red-cell serum may become attached to the erythrocytes under test. The result is that when such coated cells are suspended in saline there is no agglutination, but when they are suspended in certain human sera or dilute human serum, agglutination occurs. This is particularly the case if the human serum concerned contains proteins similar to those present in the serum of persons suffering from rheumatoid arthritis or allied conditions. In other words, the effect is akin to that which occurs in the Rose-Waaler test for the detection of rheumatoid conditions. Here one is substituting a human sensitized cell in place of a sheep sensitized cell. Such a test has actually been used by Gibson *et al.* (1956), and by Stratton and Rawlinson (1958), for this purpose. It is necessary in MN typing, therefore, to be sure that red cells are well washed and that [illegible] f any doubtful positive results [illegible] washed and the test repeated. [illegible] positive results to occur.

Anti-M and anti-N sera are notorious for their loss of specificity. They are, of course, stored frozen solid, but even under ideal storage conditions non-specific activity tends to return to the sera. The cause of this is obscure, but it is thought possible that it is due to the dissociation of antigen-antibody aggregates avulsed from the absorbing cells during serum preparation. This is the reason why the special absorption technique referred to in Chapter II is considered valuable. Multiple control tests must be put up on each occasion when blood grouping is being done to exclude this cause of error.

The P Blood Group System

Blood group P was first described by Landsteiner and Levine in 1927, at the same time as they discovered the MN groups, and the anti-P serum used was prepared in rabbits by injections of human red

cells. Anti-P serum is difficult to produce in rabbits and consequently nowadays other sources of P typing sera are used. These are either of human or animal origin. Persons are either P positive or P negative. Table 87 shows the frequency of these groups:

TABLE 87. THE P GROUPS

Group	*Genotype*	*Frequency*
P positive	{ PP { Pp	79%
P negative	pp	21%

The antibody, anti-Tj^a, and its antigen Tj^a, belong to the P system. This antibody reacts with a very high proportion of bloods so that Tj(a −) individuals are exceedingly rare. Sanger (1955) observed that all Tj(a −) individuals were P negative and found that absorption of anti-Tj^a with P negative cells gave an antibody whose reactions were identical with those of anti-P. The system is thought to be analogous to the sub-groups A_1 and A_2 and this is shown in Table 88 together with a new terminology for the P-groups which Tj^a necessitates:

TABLE 88. Tj^a AND ANTI-Tj^a SHOW THE P SYSTEM TO BE LIKE THE A_1A_2O SYSTEM
(After Sanger, 1956)

A_1A_2O		P_1P_2p	
Phenotype of cells	*Antibodies in serum*	*Phenotype of cells*	*Antibodies in serum*
O	Always anti-A + A_1	p (Tj(a −))	Always anti-P + P_1 (anti-Tj^a)
A_2 ..	Sometimes anti-A_1	P_2(P −)	Sometimes anti-P_1 (anti-P)
A_1 ..	None	P_1(P +)	None

The P antigen varies in the strength of its reaction with anti-P sera and has been divided into P-strong, P-medium and P-weak; 15 per cent of the total P positive results are of P-weak variety.

Anti-P commonly occurs in the sera of P negative people and according to Henningsen (1949), anti-P agglutinins can be found in as many as 60 per cent of unselected P negative persons. These are, on occasion, weakly active and sometimes only at low temperature. We have seen very many examples of anti-P. They are found during antenatal testing in combination with anti-D antibodies or alone, and are detected during the performance of the cross-matching test or in other serological procedures. They commonly act as saline agglutinins, active at 16° C., but are only rarely active at 37° C. The titre with P-strong cells at

16° C. varies from 1 to 128. We have observed anti-P in the serum of women at delivery a number of times but have never detected the antibody in the cord serum, so that it would appear that it does not commonly pass through the placenta. We have seen three examples of anti-P active at 37° C.; one was active in saline with a titre of 2; one was active in serum albumin alone, and the third was Case 73.

TABLE 89. TITRE OF ANTI-P (*Ra*), USING VARIETIES OF P POSITIVE CELLS

Cell sample	*Cells*	*Tube Saline agglutination, 16° C.* 1	2	4	8	16	32	64	128	256	*Control*
P+ strong	Normal	+++++	++++	+++	+	–	–	–	–	–	–
	Papainized		+++++	+++++	+++++	++++	++++	+	–	–	–
P+ weak	Normal	+	w	–	–	–	–	–	–	–	–
	Papainized		+++	+++	+	w	–	–	–	–	–
P- negative	Normal	–	–	–	–	–	–	–	–	–	–
	Papainized		–	–	–	–	–	–	–	–	–

Rosenfield and Vogel (1951) described the value of enzyme treated cells, and anti-P antibodies are very readily detected using papainized P positive red cells. There is considerable enhancement of the agglutinin titre although agglutination is not evident at 37° C. in the vast majority of cases. Table 89 shows the titration of an anti-P serum using normal and enzyme treated cells. Mollison and Cutbush (1955) have described an antibody capable of sensitizing P positive cells to an antiglobulin serum and also capable of haemolysing enzyme treated P positive cells at 37° C. in the presence of complement, and the following case showed the same unusual features.

CASE 73

Mr. T., aged 62, group A_1rr, suffering from pulmonary tuberculosis, with positive sputum, had never received any previous blood transfusions. Incompatibility was found on attempting to cross-match blood following haemoptysis and this proved to be due to a potent anti-P. Typical results are shown in Table 90.

TABLE 90. TYPICAL RESULTS WITH THE ANTI-P SERUM OF CASE 73

Cells	*Antiglobulin reagent*		*Serum albumin*	*Saline agglutination*		*Haemolysis with papainized cells*
	Anti-γ	*Anti-non-γ*	*37° C.*	*37° C.*	*16° C.*	
P-strong	–	++	+++	–	+++++	Partial.
P-medium	–	–	+	–	+++++	Trace.
P-weak	–	–	–	–	+++++	Nil.
P-negative	–	–	–	–	–	Nil.

It is important to note that this presumably dangerous antibody which was active at 37° C. and capable of causing in vitro lysis, was present in the serum of a patient who had never received any previous blood transfusions.

Anti-Tj^a, so far as is known, is present in the serum of all people who are Tj(a −).

Anti-P Grouping Sera

Various sources of anti-P sera are available. They can be prepared by absorption of certain animal sera, such as selected normal pig and horse sera; these can give reliable results. Human sera are preferable and recommended, and good ones can be obtained without too much difficulty. The titre of the serum should be as high as possible, having regard to the need to detect P weak cells (Table 89). The saline agglutination titre will increase as the temperature falls, but we are not in favour of using sera at a temperature lower than 16° C., since normal sera often contain cold antibodies active as agglutinins at 4° C., which act in a variable manner with different samples of normal red cells. The serum, therefore, should have a satisfactory titre at 16° C. and be capable of giving clear-cut, positive reactions with P-weak cells at this temperature.

Technique of P Grouping

A saline agglutination test in tubes at 16° C. is used. After incubation for 45 minutes the mixture is gently spun for 2 minutes at 500–1,000 r.p.m. The cell deposit is carefully removed with a pipette, spread out on a slide and the result is read microscopically.

It has been stated previously that anti-P antibodies are more active against enzyme treated cells and if an absolutely specific P antiserum is available this can be used at a dilution of one in two against enzyme treated cells for the purpose of determining their P group. This method is suitable for forensic blood grouping purposes. Positive and negative controls are essential; the correct positive control is a P-weak cell. False negative results in P grouping are very prone to occur due to the occurrence of the P-weak variety.

The Kell Blood Group System

In 1946, Coombs, Mourant and Race described a new blood group antigen which they called "Kell". Persons were found to be Kell-positive or Kell-negative. In 1949, Levine, Backer, Wigod and Ponder discovered anti-k, so that two antisera are known, anti-K and anti-k, and genotyping is possible within this blood group system. Anti-k is sometimes called anti-Cellano from the name of the patient in whose serum it was first discovered. The Kell blood groups and their frequencies are shown in Table 91.

TABLE 91. THE KELL GROUPS

Group	Genotype	Antisera		Frequency
		Kell (anti-K)	Cellano (anti-k)	
K+	KK	+	−	0·3%
K+	Kk	+	+	9·5%
K−	kk	−	+	90·2%

Allen *et al.* (1957) have found two other antibodies belonging to the Kell blood group system. These are anti-Kp^a giving about 2 per cent positive results, and anti-Kp^b, giving more than 99 per cent positive results. Almost all the rare Kp(b−) bloods appear to be homozygous Kp^aKp^a. The precise relationship of these antibodies to anti-K and anti-k is not yet established and even bloods which are K−k−Kp(a−b−) are known, though these seem to be exceedingly rare (Chown *et al.*, 1957).

ANTI-K AND ANTI-k

We have seen many examples of anti-Kell sera. They may occur alone in the sera of Kell-negative persons, or frequently occur in combination with other antibodies, for example anti-D+Kell, anti-Fy^a+Kell. Anti-Kell antibodies often occur in the sera of persons who have been repeatedly transfused and who are known to have produced one antibody, for example anti-E or anti-D.

Both anti-K and anti-k give rise to haemolytic transfusion reactions and to haemolytic disease of the new born.

Anti-Kell sera have been described that act as saline agglutinins, and most examples of anti-Kell will react as saline agglutinins if the layering technique is used; Table 8 (p. 33). Grove-Rasmussen, Dreisler and Shaw (1954) investigated the activity of anti-Kell sera with

TABLE 92. TITRATION OF ANTI-K (MRS G) SERUM IN VARIOUS DILUENTS

Cells	Diluent	Titre										
		1	2	4	8	16	32	64	128	256	512	Control
Kk	20% Bov alb.	−	−	−	−	−	−	−	−	−	−	−
kk		−	−	−	−	−	−	−	−	−	−	−
Kk	Serum albumin	++	++	−	−	−	−	−	−	−	−	−
kk		−	−	−	−	−	−	−	−	−	−	−
Kk	Fresh serum	+	+++	++	++	++	+	−	−	−	−	−
kk		−	−	−	−	−	−	−	−	−	−	−

special reference to their action when the sera were tested with cells suspended in whole serum and titrated in whole serum. Stratton and Dimond (1955) confirmed this and showed how inactive these sera might be in 20 per cent bovine albumin. Table 92 illustrates this.

By selecting human sera for the titration of anti-Kell and especially selecting those from certain patients suffering from rheumatoid arthritis, it is possible to make this the principal method for the detection of anti-Kell antibodies and for its use in blood grouping (Table 93).

TABLE 93. TITRATION OF ANTI-K (G) USING NORMAL AND SELECTED WHOLE SERA. SEDIMENTATION FOR TWO HOURS

Diluent	*Cells*	*Titre*											
		2	4	8	16	32	64	128	256	512	1024	2048	*Control*
Fresh serum from AB donor	Kk	+++	++	++	++	+	−	−	−	−	−	−	−
	kk	−	−	−	−	−	−	−	−	−	−	−	−
Fresh rheumatoid serum,	Kk	+++++	+++++	+++++	+++++	+++++	++++	+++	+	−	−	−	−
	kk	−	−	−	−	−	−	−	−	−	−	−	−

Most anti-Kell sera are detectable using the antiglobulin test and anti-γ-globulin reagent. Grove-Rasmussen (1956) stated that he had seen examples of anti-Kell which were detected more readily using the whole serum technique than the antiglobulin test, and Table 94 illustrates this in relation to an anti-Kell serum which had been stored frozen for many months.

TABLE 94. ANTI-K (Mi) ANTIBODY TITRATED USING VARIOUS TECHNIQUES

Method	*Cells*	*Titre*					
		1	2	4	8	16	*Control*
Selected rheumatoid serum diluent	Kk	+++++	++	+	−	−	−
	kk	−	−	−	−	−	−
Normal serum diluent . .	Kk	+	+	−	−	−	−
	kk	−	−	−	−	−	−
Routine antiglobulin test . .	Kk	−	−	−	−	−	−
	kk	−	−	−	−	−	−

Papainized red cells are not satisfactory for detecting anti-K antibodies. Dosage effects of K and k antigens are not very clearly

demonstrable, but are best shown using the whole serum agglutination technique (Table 95) rather than by the antiglobulin test.

Anti-k sera occur only very rarely and we have seen one example (Case 68, p. 262).

TABLE 95. DOSAGE EFFECT IN KELL SYSTEM DEMONSTRATED USING ANTI-K (G) AND ANTI-k (Ho), WITH WHOLE SERUM AGGLUTINATION TECHNIQUE

Cells	Serum	*Whole serum diluent Titre*										Score
		1	2	4	8	16	32	128	256	512	*Control*	
O, rr, KK	Anti-K	+++++	+++++	+++++	+++++	++++	++	+	−	−	−	27
	Anti-k	−	−	−	−	−	−	−	−	−	−	0
O, rr, Kk	Anti-K	+++++	+++++	+++++	+++++	++	−	−	−	−	−	22
	Anti-k	+++++	+++++	++++	++	−	−	−	−	−	−	16
O, rr, kk	Anti-K	−	−	−	−	−	−	−	−	−	−	0
	Anti-k	+++++	+++++	+++++	++++	++	−	−	−	−	−	21

TECHNIQUES OF KELL GROUPING

Two techniques are useful: an agglutination technique using cells suspended in serum (preferably specially selected serum) and the antiglobulin technique. Controls are needed and heterozygous Kk cells should always be used as the positive control in both anti-K and anti-k grouping tests.

The Lewis Blood Group System

THE LEWIS ANTIGENS

The Lewis blood groups were discovered in 1946 by Mourant, who found the anti-Lewis antibody in a human serum. This was subsequently known as anti-Le^a. Andresen (1948a) described another kind of anti-Lewis antibody, subsequently known as anti-Le^b. Two antigens were therefore detectable on the red cells using these antisera and a number of blood groups defined. The Lewis groups are detailed in Table 96.

TABLE 96. LEWIS GROUPS USING ANTI-Le^a AND ANTI-Le^b

Phenotype	*Possible genotypes*	*Frequency (Gp. O) (percentage)*
Le(a+b−)	Le^aLe^a	22
Le(a−b+)	Le^aLe^b Le^bLe^b Le^bLe^o	72
Le(a−b−)	Le^aLe^o Le^oLe^o	6

Andresen (1948b) observed that parents of Le(a+) persons could both be Le(a−) and therefore suggested that Le(a+) was a recessive character. This is now generally accepted. Consequently Le(a+) is not recognizable as such in the heterozygous genotype Le^aLe^b (in adults). Cutbush, Giblett and Mollison (1956) suggest that if an incomplete anti-Le^a is used to sensitize ficin treated Le(b+) cells to an antiglobulin serum it will be found that some bloods of groups O and A_2 which seemed to be Le(a−b+) by the usual tests appear to belong to the phenotype Le(a+b+).

Andresen and Jordal (1949) have described a further Lewis antibody, anti-X, and have introduced a further system, X-x. Persons who are xx appear to be Le(a−b−). It may well be that anti-X is a mixture of anti-Le^a and anti-Le^b. In certain sera these two antibodies are equally strongly active and in one sample we have succeeded in separating the two component parts and isolating anti-Le^a.

Sneath and Sneath (1955) found that cells could lose the Le^a or Le^b antigen when incubated at 35° C. for 24 hours in plasma lacking the antigen. Moreover, cells incubated in plasma containing the antigens could take them up and change their Lewis groups accordingly, so that, for example, cells reacting as Le(a+b+) could be produced. These changes are difficult to produce in *vitro*, but they might explain why certain anti-Le^a and anti-Le^b sera appear to react with cells of type Le(a−b−) quite apart from their ability to detect heterozygotes.

It has also been found that excessive washing of Le(a+b−) cells in saline tends to remove the Le^a antigen from the cell surface (Grubb and Morgan, 1949). This is sometimes a difficulty when using cells

TABLE 97. RESULTS OF TESTS ON 10 SAMPLES OF GROUP O CORD CELLS

Normal cord sample No		*Anti-Le^a (Mg) saline agglutination*	*Anti-Le^a (E) antiglobulin test*	*Anti-Le^b (M) saline agglutination*	*Anti-Le^b (Ma) antiglobulin test*
1		+	++++	−	−
2		−	−	−	−
3		−	−	−	−
4		−	+	−	−
5		−	−	−	−
6		−	−	−	−
7		−	−	−	−
8		−	−	−	−
9		−	−	−	−
10		−	−	−	−
Adult normal cells	Le(a+b−)	+++++	+++++	−	−
	Le(a−b+)	+	−	+++	+++++
	Le(a−b−)	−	−	−	−

preserved by freezing in glycerol-citrate, since the recovery process involves multiple washings.

Other interesting features concerning the Lewis blood groups should be noted. These are:

1. The effect of age on the Lewis blood groups of the red cell. Rosenfield and Ohno (1954) stated that all cord cells are Le(a−b−) and this is largely our experience. Using anti-Le^a and anti-Le^b potent saline agglutinins, we find an occasional sample positive with anti-Le^a alone. Cutbush *et al.* (1956) report that using incomplete anti-Le^a in the antiglobulin test over 50 per cent of the cord samples showed the presence of Le^a antigen. We have examined cord cells with potent anti-Le^a and anti-Le^b antibodies and some results are detailed in Table 97.

2. Andresen (1948b) showed that, using anti-Le^a serum, the percentage of Le(a+) results was higher in babies up to 1 or 2 years of age than in adults (Table 98).

TABLE 98. EFFECT OF AGE ON Le(a+) FREQUENCY (after Andresen)

	1–3 *Months*	4–6 *Months*	7–9 *Months*	10–12 *Months*	*Adults*
Le(a+) percentage ..	79	73	36	29	21

Andresen concluded that receptors are weak at birth and the preponderance of Le^a is due to a tardy development of Le^b.

3. The results obtained when using anti-Le^b typing serum depend upon the ABO group of the person. Andresen (1948a) states that the frequency of Le^b is diminished in A_1 persons; "the mere presence of the character A_1 or corresponding gene inhibits the development of the receptor Le^b which corresponds to the phenomenon in genetics designated epistasis" (Table 99).

TABLE 99. EFFECT OF ABO GROUPS ON LEWIS TYPING (after Andresen 1948a)

ABO group	*Le(b+) (percentage)*	*Le(b−) (percentage)*
A_1	41·8	58·2
A_2	70·0	30·0
O	72·0	28·0

Anti-Le^b sera have frequently been described (Brendemoen, 1950), which give the expected results with A_1 cells.

4. Grubb (1948) has observed a very close relationship between the

ability to secrete ABH substances in saliva and the groups Le(a+) and Le(a−). All Le(a+) persons are non-secretors and nearly all Le(a−) persons are secretors (p. 127).

5. If Lewis grouping is carried out on the red cells during pregnancy an excessive number of persons will be found who are of type Le(a−b−). Brendemoen (1952) found this and we have seen it also.

The Lewis Antibodies

Anti-Le^a is commonly found as a naturally occurring antibody. Kissmeyer-Nielsen, Bastrup-Madsen and Sternderup (1935) from an extensive survey considered that the incidence was 1 in 300. We have observed examples in tests using papainized red cells on normal sera of all ABO groups. Anti-Le^a may occur alone in the serum or very often in combination with anti-Le^b. Sometimes both antibodies may be equally active, giving rise to sera containing an antibody reacting positively with approximately 90 per cent of group O cells. Anti-Le^b unaccompanied by anti-Le^a is less frequent and may occur alone in the serum or together with anti-H.

Lewis antibodies have certain characteristic features, as follows:

1. They occur in normal sera, very often without evidence of previous immunization by blood transfusion or pregnancy.

Case 74

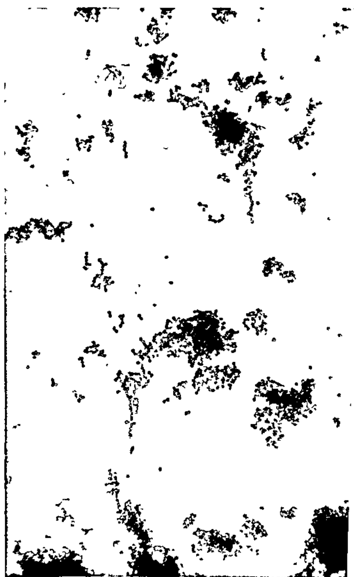
N.C., a girl aged 12 years, suffering from bronchiectasis, was admitted to hospital for lobectomy. She had not previously received blood transfusions or intramuscular injections of blood. Cross-matching of blood preparatory to operation revealed *incompatibility* and the serum was referred to us. It contained potent anti-Le^a and anti-Le^b antibodies. She was A_1 R_1r, Le(a−b−) and was successfully transfused with A_1, Rh positive, Le(a−b−) blood. (Table 100.)

Table 100. Results of Testing Serum in Case 74 prior to Transfusion

Test cells	*Saline agglutination* 16° C.	37° C.	*Haemolysis using papainized cells*	*Antiglobulin test*
Le(a+b−) ..	+++++	+++	Present	++++
Le(a−b+) ..	+++++	+	Present	+++
Le(a−b−) ..	—	—	Absent	—
Le(a−b−) .	—	—	Absent	—
Own	—	—	Absent	—

2. Activity as saline agglutinins. Most Lewis antibodies will agglutinate red cells in saline. Anti-Le^a will agglutinate Le(a+) cells in saline at 16° C. and at 37° C. Usually, but not always, the reactions are stronger at 16° C. and may be very weak or absent at 37° C.

PLATE XVI.



Agglutination due to anti-Le^a showing chains of cells
See p 295 × 220

Often anti-Le^a antibodies are much less readily detected as saline agglutinins if the serum has been inactivated by heating at 56° C. for 30 minutes than when using fresh serum (Table 101), but again the reverse can occur. Using the tube technique, saline agglutination may show certain characteristic features (Stratton, 1956).

(*a*) Reversal of agglutination when the clumped and agglutinated cells are spread out on a slide; and

(*b*) Characteristic appearance of the agglutinates.

TABLE 101. EFFECT OF HEAT INACTIVATION ON SALINE AGGLUTINATION TITRE OF ANTI-Le^a SERUM

Anti-Le^a	*Temperature ° C.*	*Titre*						
		1	2	4	8	16	32	*Control*
Fresh serum	16	+++++	++	+	w	–	–	–
	37	++++	+++	+	–	–	–	–
Inactivated serum	16	+++++	+	–	–	–	–	–
	37	+++	+	–	–	–	–	–

The term "reversal" rather than "dispersal" is used because the phenomenon was first observed with an anti-Le^a which gave a positive result on an open slide using saline suspended Le(a+) cells. This agglutination reached a maximum in a minute or two and then slowly dispersed. The speed of reversal of agglutination varies; it may occur in a few minutes or in less than a minute, so that while the clumps are observed on a slide and show the typical Lewis agglutination formation it will be seen to melt away and evenly suspended cells remain. On the other hand, no reversal may occur and firm agglutinates be present as long as the slide is observed. It is, therefore, only certain examples of anti-Le^a sera that show the reversal phenomenon. Reversal is thus an active process. *Such sera are unsuitable for use as typing sera.*

The Lewis agglutination is seen in Plate XVI, and the chains of cells linked together are characteristic of this type of agglutination.

On certain films an agglutination resembling streptococcal bacterial chains is seen; multiple small chains of cells. The agglutinates are often extremely fragile and break up on agitating the slide quite unlike the firm clumps seen with antibodies of other specificities. Occasionally the appearance may be mistaken for rouleaux formation.

3. The haemolytic activity. Rosenfield and Vogel (1951) described anti-Le^a and anti-Le^b haemolysins. Anti-Le^b acts as a haemolysin less frequently than anti-Le^a. Lysis may be observed using normal red cells but it is much more readily detected, as Rosenfield and Vogel (1951) showed, if enzyme treated cells are used. Papainized red cells are recommended. Table 102 shows the frequency of occurrence of

haemolysins in a number of anti-Le^a and anti-Le^b sera. The haemolysis is dependent on the presence of complement.

TABLE 102. THE HAEMOLYTIC ACTIVITY OF ANTI-LEWIS ANTIBODIES

Antibody	*Number with haemolysins to normal and enzyme cells*	*Number with haemolysins to enzyme cells only*	*Number with no haemolysins*	*Total*
Anti-Le^a ..	7	6	3	16
Anti-Le^b ..	1	2	3	6

4. The dependence of certain Lewis antibodies on the presence of fresh serum for their detection using the antiglobulin test. It has now been observed by many workers that Lewis sera may give a positive antiglobulin test. It was found that the antiglobulin test needed fresh serum (Mollison and Cutbush, 1955) (p. 57). Certain sera, especially when freshly collected, give a positive antiglobulin test with the anti-γ-globulin reagent, but many will not react positively in the antiglobulin test unless an anti-non-γ-globulin reagent is used. It was also suggested that sera would not give a positive antiglobulin test unless fresh serum was present during their sensitization. Vaughan and Waller (1957) subsequently showed that when Le(a+) cells were sensitized with inactivated anti-Le^a serum they gave a negative antiglobulin test, but if the washed sensitized cells were suspended in fresh antibody free serum and re-washed, the test became positive (p. 57). Typical results are shown in Table 103. The problem is further discussed in Chapter III.

TABLE 103. ANTIGLOBULIN TEST WITH ANTI-Le^a AND ANTI-Le^b SERA

Serum	*State*	*Antiglobulin test*		
		anti-non-γ-globulin reagent	*anti-γ-globulin reagent*	*Control*
Anti-Le^a .	Fresh serum	+++++	Neg.	Neg.
	Heat inactivated	Neg.	Neg.	Neg.
Anti-Le^b	Fresh serum	+++++	Neg	Neg.
	Heat inactivated	Neg.	Neg.	Neg.

5. Reaction with enzyme treated test cells (Rosenfield and Vogel, 1951). Papain treated test cells may be used for the detection of anti-Le^a and anti-Le^b sera, and there is enhancement of activity. The titre using such cells at 16° C. is greater than that using normal cells (Table 104). Most antisera are optimally active at 16° C., and using the papain cell slide technique it is quite possible to detect anti-Le^a and anti-Le^b antibodies,

though some of the weaker ones may be difficult to detect using this method. With occasional sera papainization of the cells offers no advantage and the alternative methods of saline agglutination and the antiglobulin test should be employed.

TABLE 104. ANTI-Le^a AND ANTI-Le^b TITRATED WITH NORMAL AND PAPAINIZED RED CELLS AT 16° C.

Serum	*Test*	*Cells*	*Titre* 1	2	4	8	16	32	64	*Control*
Anti-Le^a	Slide agglutination	N	–	–	–	–	–	–	–	–
		P		w	–	–	–	–	–	–
	Tube agglutination	N	+++++	+++	+	w	–	–	–	–
		P		+++++	++	+	–	–	–	–
Anti-Le^b	Slide agglutination	N	+	w	–	–	–	–	–	–
		P		+++++	+++	+	–	–	–	–
	Tube agglutination	N	+++++	+++	+	–	–	–	–	–
		P		+++++	+++	++	+	–	–	–

N = normal cells. P = papainized cells.

We have found both anti-Le^a and anti-Le^b associated with haemolytic transfusion reactions, but their role in the causation of haemolytic disease of the newborn is doubtful. We have seen anti-Le^a pass the placental barrier, but the infant's direct Coombs test was negative, possibly because the infant's Lewis antigens were not developed at birth.

CASE 75

Mrs. E.H., group A, rr, Le(a–b–), was found to have anti-Le^a +Le^b in her serum ... other antibodies were detect ... –b+). When her child was ... and the cells reacted as Le(a–b–). The direct Coombs test was negative using both anti-γ-globulin and anti-non-γ-globulin reagents. The maternal and cord sera gave the results shown in Table 105. The infant was not ... 16 gm. per cent. so, but there wa ... 8·5 gm. per cen ... any treatment and the role of anti-Le^a in this case was undecided.

ANTI-LEWIS GROUPING SERA

Anti-Le^a for use as *typing* sera should preferably be sought in persons of group AB, since absorption should be avoided where

TABLE 105. REACTIONS OF THE MATERNAL AND CORD SERUM OF CASE 75 WITH CELLS OF DIFFERENT LEWIS GROUPS

Method of test	*Mother's serum**			*Cord serum**		
	Le (*a*+*b*−)	*Le* (*a*−*b*+)	*Le* (*a*−*b*−)	*Le* (*a*+*b*−)	*Le* (*a*−*b*+)	*Le* (*a*−*b*−)
Antiglobulin						
anti-γ ..	+++	−	−	+	−	−
anti-non-γ ..	+++++	−	−	−	−	−
Saline agglutination 37° C.	+++++ H.	−	−	−	−	−
Saline agglutination 16° C.	+++++	++	−	++	−	−
Papainized cells (slide test) ..	++++	++	−	+++	−	−
Haemolysis with papainized cells at 37° C. ..	75%	0%	0%	0%	0%	0%

H = haemolysis. * = Fresh serum.

possible. If this is not possible, persons of other groups can have their serum suitably absorbed. Most anti-Le^a sera suitable for use as grouping sera are optimally active as saline agglutinins at 16° C. Serum should be selected of high avidity and as good a titre as possible, and it should give a steady and firm agglutination and show no tendency towards agglutination reversal. The anti-Le^a sera should be inactive against cells of Le(a−b+) and cells Le(a−b−). Many anti-Le^a sera contain traces of anti-Le^b. It may be possible to remove this by absorption, but it may be difficult to do so and it is desirable to avoid absorption of these sera as much as possible.

Secretor saliva cannot, in general, be used to neutralize anti-A and anti-B in Lewis antisera, since most salivas contain Le^a or Le^b substances.

Anti-Le^b sera suitable for use as typing sera are more difficult to obtain. They are much less common in occurrence than anti-Le^a and the serum may contain other antibodies or have apparently non-specific activity. Anti-H or anti-O are common additional antibodies. Much confusion has arisen in the past owing to the fact that anti-H antibodies may have activities which resemble those of anti-Le^b. Anti-Le^b typing sera should have the following characteristics:

1. They should act as saline agglutinins with an optimum temperature of 16° C. Temperatures lower than this should be avoided.
2. They should not react with group O, Le(a+b−) cells. Several examples of this group should be tested.

3. Sera may be found which, although possessing properties (1) and (2) above, will react weakly, or not at all, with many cell samples belonging to group A_1. A serum should be selected which reacts with as many Le(a−b+) samples of this group as possible.

When testing the sera of adults we have confined our blood grouping to the use of potent saline agglutinins and have not thought it necessary to use incomplete anti-Le^a although this has been used experimentally on many occasions. Nor have we routinely used incomplete anti-Le^a for testing cord cells, although in special cases it might be desirable to do this; for example, where a suspicious case of haemolytic disease of the newborn is associated with maternal anti-Le^a.

Some examples of both anti-Le^a and anti-Le^b sera are difficult to preserve frozen solid, since their avidity and titre fall. In selecting grouping sera, therefore, it is desirable to select those which retain their avidity and potency when stored frozen solid. A better system is to have donors whose sera are known to contain anti-Le^a and anti-Le^b and whose blood can be collected at intervals. If such sera have been obtained following incompatible blood transfusion they may lose their avidity and titre with the passage of time. Very often, for example, they lose their ability to act as haemolysins.

TECHNIQUE OF LEWIS GROUPING

Saline agglutination techniques at 16° C. are generally the best and the strength and firmness of the agglutinates are much improved by centrifugation. Incubation for 30–45 minutes at 16° C., followed by centrifugation at 500–1,000 r.p.m. for two minutes, will generally give satisfactory results. Controls are essential and Le(a+b−), Le(a−b+) and Le(a−b−) should always be included together with suitable absorption controls where necessary.

Two causes of false negative results particular to Lewis grouping arise from the instability of the sera and from the loose and unstable character of the [illegible]
[illegible]
[illegible] in reading the results will minimize the latter.

The Lutheran Blood Group System

In 1946, Callender and Race defined a new antigen on the red cells which they called "Lutheran". The antiserum was anti-Lu^a. In 1956, Cutbush and Chanarin described the finding of anti-Lu^b. This makes possible genotyping in the Lutheran blood group system (Table 106).

We have observed three examples of anti-Lu^a. In the first case the anti-Lu^a antibody was active as a saline agglutinin at 16° C. but not at 37° C. Its titre was 2. It was detected during the performance of a cross-matching test. The second case was also found during cross-

TABLE 106 LUTHERAN GROUPS

Group	Phenotype notation	Genotypes	Frequency
Lutheran positive ..	Lu(a+b−)	Lu^aLu^a	0 15%
	Lu(a+b+)	Lu^aLu^b	7%
Lutheran negative ..	Lu(a−b+)	Lu^bLu^b	93%

matching and occurred in a male patient who had received many transfusions for myeloid leukaemia. It was active as a saline agglutinin at 16° C. and 37° C. The third case is Case 71 described on p. 267, in which the anti-Lu^a antibody was present with anti-E and anti-H in the serum. This antibody was unusual in that it gave a positive antiglobulin test with Lu(a+) cells and was active against serum albumin suspended cells (Tables 107 and 80).

TABLE 107. TESTS ON ANTI-Lu^a SERUM OF CASE 71

Method	Cells	Titre 1	2	4	8	16	Control
Saline agglutination 16° C.	Lu(a+)*	++++	+	w	−	−	−
37° C.		w	−	−	−	−	−
Antiglobulin test:							
(i.) anti-γ reagent . ..	Lu(a+)	++					
	Lu(a−)	−					
(ii) anti-non-γ reagent ..	Lu(a+)	++					
	Lu(a−)	−					

* Lu(a−) cells gave negative results.

These Lutheran antibodies, often detected as saline agglutinins at 16° C., have mostly been of low titre and difficult to detect. Rosenfield (1957) has recently detected an anti-Lu^a saline agglutinin having a titre of 256 at 16° C.

TECHNIQUE OF LUTHERAN GROUPING

The saline agglutination technique at 16° C. is used. The cell deposit is removed carefully and examined under the microscope. The agglutination caused by anti-Lutheran antibodies often, but not always, consists of small clumps of agglutinated red cells in a field containing a large number of free cells. It is important to examine the cells carefully when carrying out Lutheran typing, otherwise this characteristic agglutination may be missed. Positive and negative controls are needed. The Lu^a antigen shows some variation in strength and if possible a weak reactor should be used for the positive control.

The Duffy Blood Group System

The Duffy blood group was described by Cutbush, Mollison and Parkin in 1950 and they called the antibody anti-Fy^a. Subsequently, Ikin, Mourant, Pettenkofer and Blumenthal (1951) described anti-Fy^b. Using these two antibodies, genotyping in this group is possible (Table 108).

TABLE 108. DUFFY GROUPS

Group	*Phenotype*	*Genotype*	*Frequency*
Duffy positive	Fy(a+b−)	Fy^{a}Fy^a	17%
	Fy(a+b+)	Fy^{a}Fy^b	49%
Duffy negative	Fy(a−b+)	Fy^{b}Fy^b	34%

Sanger, Race and Jack (1955) found that a high proportion of New York Negroes reacted as Fy(a−b−), so that a third gene appears to be involved in the Duffy blood group system, though this is not found in Caucasians.

We have encountered four examples of anti-Fy^a serum. All persons in whose serum this antibody was found had received blood transfusions, many of them multiple blood transfusions. In one case, the antibody was detected during the course of the cross-matching test. In two of the other cases, blood which had been given had resulted in signs of haemolytic reaction, i.e. there had been jaundice or anuria. In two of the cases, anti-Fy^a was the only antibody present in the serum. In the third case it was associated with anti-K and anti-Le^a and in the fourth with anti-K. All these examples of anti-Fy^a acted only in the antiglobulin test, one of them acting both with anti-γ and anti-non-γ-globulin reagents. Anti-Fy^a has been described as active as a saline agglutinin and Race, Sanger and Lehane (1953) have used such a serum to demonstrate "a clear dosage effect of the Fy^a gene". Differences of antigen strength between individuals of the same genotype were also found. Such sera often seem to lose their ability to agglutinate Fy(a+) cells in saline following storage.

TECHNIQUE OF DUFFY GROUPING

The antiglobulin test is used. Care should be taken to see that the dose of test cells and strength of anti-globulin reagent are suitable for the particular anti-Fy^a serum.

Controls are required and positive controls need careful selection. The strength of antigen varies from one individual to another (*vide supra*) and a weak positive reactor should be used.

The Kidd Blood Group System

Anti-Jk^a antibody was described by Allen, Diamond and Niedzeila (1951), who defined the antigen Jk^a. Subsequently, in 1953, Plaut,

Ikin, Mourant, Sanger and Race described anti-Jk^b and genotyping is possible within this group. The groups and their frequencies are shown in Table 109, which is based on our own finding of 327 Jk(a+) individuals in a total of 400 group O and A donors tested.

TABLE 109. KIDD GROUPS

Group	*Genotype*	*Frequency*
Jk(a+b−) ..	Jk^aJk^a	32·8%
Jk(a+b+) ..	Jk^aJk^b	48·9%
Jk(a−b+) ..	Jk^bJk^b	18·3%

We have seen three examples of anti-Jk^a antibody. One of these was a weak antibody but the second was stronger and associated with a haemolytic transfusion reaction. The third was Case 70 (p. 265). These sera were active only when the antiglobulin test was used. In the second case, although the fresh serum reacted weakly with anti-γ-globulin reagents, the stored serum would only react with anti-non-γ-globulin reagents and only with these if fresh serum was added when the cells were sensitized.

Fresh serum must always be present during the sensitization of the cell or subsequently added for a reasonably strong positive antiglobulin test to be obtained. The papainized cell Coombs test will give a higher titre with anti-Jk^a serum than when normal cells are used. Here, also, fresh serum should be present. Sera may occasionally react as saline agglutinins as did the original example described by Allen *et al.* (1951).

TECHNIQUE OF KIDD GROUPING

The technique used is the antiglobulin test with fresh serum added in the first part of the test.

Many anti-Jk^a sera deteriorate on storage and in the authors' experience no method of storage has been found which will prevent this. This does not appear to be solely due to the deterioration of fresh serum factors, such as complement. After a short period of storage, activity can be restored by the addition of fresh serum and even older samples may be used by employing the enzyme cell Coombs test, but eventually the serum becomes useless as a grouping serum.

Controls should include Jk(a−) cells and a weakly reacting Jk(a+) cell. *The Jk^a antigen varies in strength like the Fy^a antigen,* so that similar considerations apply.

Other Blood Group Systems

A number of blood group antibodies, other than those already mentioned, are known (Table 110). Some of these have been sufficiently extensively investigated for it to be said that they are not connected

with any of the blood group systems described previously, but others have not been adequately investigated, and it is possible that they are related to one another, or to the other systems. Some are of apparently natural occurrence in the serum and act as saline agglutinins; others are immune antibodies active in the antiglobulin test and causing haemolytic disease of the newborn. Two of them, anti-Vel and anti-Yt[a], give more than 99 per cent of positive results; the remainder give less than 1 per cent of positive results and in many cases the positive results so far found are confined to the members of one family. These are the so-called "family" antigens, but it is likely that other positive results could be found in many cases if more extensive tests could be carried out, and particularly if they could be carried out on the blood of

TABLE 110. FAMILY ANTIGENS AND OTHER RARE BLOOD GROUPS

Name	*Frequency*	*Optimal mode of reaction*	*Discoverer*
Vel	99 96%	Agglutination and haemolysis	Sussman and Miller, 1952.
Yt[a] ..	99 62%	Modified antiglobulin	Eaton *et al.*, 1956.
Diego (Di[a])	Very low in Caucasians; higher in certain other races	Antiglobulin	Layrisse *et al.*, 1955.
Js	Ditto	Antiglobulin	Giblett, 1958.
Chr_a ..	0 4%	Albumin agglutination 37° C.	Kissmeyer-Nielsen, 1955.
Wright (Wr[a])	0 3%	Various	Holman, 1953.
Becker ..	Positives confined to a single family in each case	Antiglobulin	Elbel and Prokop, 1951.
Berrens (Be[a])		Antiglobulin	Davidsohn *et al.*, 1953.
Batty (By[a])		Antiglobulin	Simmons and Were, 1955.
Cavaliere (Ca)		Antiglobulin	Wiener and Brancato, 1953.
Graydon ..		Saline agglutination, 16° C.	Graydon, 1946.
Levay ..		Saline agglutination, 37° C.	Callender and Race, 1946.
Romunde (Rm) ..		Antiglobulin	van der Hart *et al*, 1954.
Ven ..		Antiglobulin	van Loghem *et al.*, 1952.

(See also Table 111)

individuals of different races. Anti-Diego (anti-Di^a) is an example of this. It was at first thought to define a family antigen and has given negative results with a large number of Caucasian bloods, but it is now known to give up to 36 per cent positive results with the blood of some South American Indian tribes and certain other races. Anti-Wr^a is another interesting antibody which was at first thought to define a family antigen. It is now known, however, that Wr(a+) individuals can be found in the general population, though these are very rare. An interesting feature of the Wright blood group system is that the antibody, anti-Wr^a, is present as a naturally occurring antibody in the serum of many normal individuals (Dunsford, 1954). We tested 632 normal donors and detected 20 examples of anti-Wr^a. This antibody has been overlooked for a long time because its existence can only be demonstrated if one has Wr(a+) cells to test the sera with. A similar situation occurs with anti-Mi^a and anti-Vw, two antibodies of the MNS system. Here again, the antigens concerned are very rare, but the antibodies are not uncommon and are, apparently, naturally occurring. The behaviour of antigens and antibodies of this type is certainly intriguing and makes us wonder how far our knowledge of the other blood group systems falls short of the truth.

References

Allen, F. H., Diamond, L. K., and Niedziela, Bevely (1951). A new blood-group antigen. *Nature*, 167, 482.

Allen, F. H., and Lewis, Sheila J. (1957). Kp^a (Penny), a new antigen in the Kell blood group system. *Vox Sang.*, 2, 81.

Andresen, P. H. (1947). Reliability of the exclusion of paternity after the MN and ABO systems as elucidated by 20,000 mother-child examinations and its significance in the medico-legal conclusion. *Acta Path. Microbiol. Scand.*, 24, 545.

Andresen, P. H. (1948a). The blood group system L. A new blood group L_2. A case of epistasy within the blood groups. *Acta Path. Microbiol. Scand.*, 25, 728.

Andresen, P. H. (1948b). Blood group with characteristic phenotypical aspects. *Acta Path. Microbiol. Scand.*, 24, 617.

Andresen, P. H., and Jordal, K. (1949). An incomplete agglutinin related to the L-(Lewis) system. *Acta Path. Microbiol. Scand.*, 26, 636.

Brendemoen, O. J. (1950). Further studies of agglutination and inhibition in the Le^a-Le^b system. *J. Lab. Clin. Med.*, 36, 335.

Brendemoen, O. J. (1952). Some factors influencing Rh immunization during pregnancy. *Acta Path. Microbiol. Scand*, 31, 579.

Callender, Sheila T., and Race, R. R. (1946). A serological and genetical study of multiple antibodies found in response to blood transfusion by a patient with lupus erythematosus diffusus. *Ann. Eugen.*, 13, 102.

Chalmers, J. N. M., Ikin, Elizabeth W., and Mourant, A. E. (1953). A study of two unusual blood-group antigens in West Africans. *Brit. Med. J.*, ii, 175.

Chown, B., Lewis, Marion, and Kaita, Kiroko (1957). A "new" Kell blood-group phenotype. *Nature*, 180, 711.

Coombs, R. R. A., Mourant, A. E., and Race, R. R. (1946). In vivo iso-sensitization of red cells in babies with haemolytic disease. *Lancet*, i, 264.

Cutbush, Marie, Mollison, P. L., and Parkin, Dorothy (1950). A new human blood group. *Nature*, 165, 188.

Cutbush, Marie, and Chanarin, I. (1956). The expected blood group antibody anti-Lu^{b}. *Nature*, 178, 855.

Cutbush, Marie, Giblett, Eloise R., and Mollison, P. L. (1956). Demonstration of the phenotype Le(a+b+) in infants and adults. *Brit. J. Haem.*, 2, 210.

Davidsohn, I., Stern, K., Strauser, E. R., and Spurrier, Wilma (1953). Be, a new "private" blood factor. *Blood*, 8, 747.

Dunsford, I. Ikin, Elizabeth W., and Mourant, A. E. (1953). A human blood group gene intermediate between M and N. *Nature*, 172, 688.

Dunsford, I. (1954). The Wright blood group system. *Vox Sang.*, 4, 160.

Eaton, B. R., Morton, J. A., Pickles, Margaret M., and White, K. E. (1956). A new antibody Yt^{a} characterizing a blood-group antigen of high incidence. *Brit. J. Haem.*, 2, 333.

Elbel, H., and Prokop, O. (1951). Ein neues erbliches Antigen als Ursache gehäufter Fehlgeburten. *Zeitschr. f. Hygiene*, 132, 120.

Giblett, Eloise R. (1958). Js, a "new" blood-group antigen found in Negroes. *Nature*, 181, 1221.

Gibson, H. J., and Ling, N. R. (1956). Modified Waaler-Rose reaction employing sensitized human cells. *Ann. Rheum. Dis.*, 15, 246.

Graydon, J. J. (1946). A new rare isohaemagglutinogen. *Med. J. Austral.*, 2, 9.

Greenwalt, T. J., Sasaki, T., Sanger, Ruth, Sneath, Joan, and Race, R. R. (1954). An Allele of the S(s) blood group genes. *Proc. Nat. Acad. Sci.*, 40, 1126.

Grove-Ra[illegible] A serologic [illegible] sis on cross- [illegible] detect incompatibility due to anti-Kell antibodies as well as anti-A, anti-B and Rh antibodies. *Am. J. Clin. Path.*, 24, 1211.

Grove-Rasmussen, M. (1956). Personal communication.

Grubb, R. (1948). Correlation between Lewis blood group and secretor character in Man. *Nature*, 162, 933.

Grubb, R., Morgan, W. T. J. (1949). The "Lewis" blood group characters of erythrocytes and body fluids. *Brit. J. Exp. Path.*, 30, 198.

Hart, Mia van der, Bosman, Hélène, and Loghem, J. J. van (1954). Two rare human blood group antigens. *Vox Sang.*, 4, 108.

Henningsen, K. (1949). Investigations on the blood factor P. *Acta Path. Microbiol. Scand.*, 26, 639.

Holman, C. A. (1953). A new rare blood-group antigen (Wr^{a}). *Lancet*, ii, 119.

Ikin, Elizabeth W., Mourant, A. E., Pettenkofer, H. J., and Blumenthal, G. (1951). Discovery of the expected haemagglutinin, anti-Fy^{b}. *Nature*, 168, 1077.

Jakobowicz, Rachel, Bryce, Lucy M., and Simmons, R. T. (1949). The occurrence of unusual positive Coombs reactions and M variants in the blood of a mother and her first child. *Med. J. Australia*, **2**, 945.

Kissmeyer-Nielsen, F. (1955). A new rare blood group antigen: Chr^{a}. *Vox Sang.*, **5**, 102.

Kissmeyer-Nielsen, F., Bastrup-Madsen, Kirsten, and Stenderup, A. (1955). Irregular blood-group antibodies. Incidence and clinical significance. *Dan. Med. Bull.*, **2**, 202.

Landsteiner, K., and Levine, P. (1927a). A new agglutinable factor differentiating individual human bloods. *Proc. Soc. Exp. Biol. and Med., N.Y.*, **24**, 600.

Landsteiner, K., and Levine, P. (1927b). Further observations on individual differences of human blood. *Proc. Soc. Exp. Biol. and Med. N.Y.*, **24**, 941.

Landsteiner, K., Strutton, W. R., and Chase, M. W. (1934). An agglutination reaction observed with some human bloods, chiefly among Negroes. *J. Immunol.*, **27**, 469.

Layrisse, M., Arends, T., and Dominquez Sisco, R. (1955). Nuevo grupo sanguineo encontrado en descendientes de Indios. *Separata de Acta Medica Venezolana*, **3**, 132.

Levine, P., Backer, May, Wigod, M., and Ponder, Ruth (1949). A new human hereditary blood property (Cellano) present in 99.8% of all bloods. *Science*, **109**, 464.

Levine, P, Kuhmichel, A. B., Wigod, M., and Koch, Elizabeth (1951). A new blood factor, allelic to S. *Proc. Soc. Exp. Biol. and Med., N.Y.*, **78**, 218.

Levine, P., Stock, A. H., Kuhmichel, A. B., and Bronikovsky, N. (1951). A new human blood factor of rare incidence in the general population. *Proc. Soc. Exp. Biol. and Med.*, **77**, 402.

Loghem, J. J. van, and Hart, Mia van der (1952). Een zeldzaam voorkomende bloedgroep (Ven). *Bull van het Centraal Lab. van de Bloedtrans. van het Nederlandse Rode Kruis*, **2**, 225.

Miller, E. B., Rosenfield, R. E., Vogel, P., Haber, Gladys, Gibbel, Natalie (1954). The Lewis blood factors in American Negroes. *Am. J. Phys. Anthrop.*, **12**, 427.

Mollison, P. L., and Cutbush, Marie (1955). The use of isotope labelled red cells to demonstrate incompatibility in vivo. *Lancet*, **i**, 1290.

Mourant, A. E. (1946). A "new" human blood group antigen of frequent occurrence. *Nature*, **158**, 237.

Plaut, Gertrude, Ikin, Elizabeth W., Mourant, A. E., Sanger, Ruth, and Race, R. R. (1953). A new blood-group antibody, anti-Jk^{b}. *Nature*, **171**, 431.

Race, R. R., Sanger, Ruth, and Lehane, D. (1953). Quantitative aspects of the blood-group antigen, Fy^{a}. *Ann. Eugen.*, **17**, 255.

Race, R. R., and Sanger, Ruth (1954). *Blood Groups in Man.* 2nd Ed. Oxford: Blackwell Scientific Publications.

Rosenfield, R. E., and Vogel, P. (1951). The identification of hemagglutinins *Trans. N.Y. Acad. Sci.*, **13**, 213.

e (1954). Unpublished observations

Rosenfield, R. E. (1957). Personal communication.

Sanger, Ruth (1955). An association between the P and Jay systems of blood groups. *Nature*, 176, 1163.

Sanger, Ruth (1956). The P and Jay systems of blood groups. Boston: VIth Congress of the International Soc. of Blood Transf.

Sanger, Ruth, Race, R. R., and Jack, J. (1955). The Duffy blood groups of New York Negroes: the phenotype Fy(a−b−). *Brit. J. Haemat.*, 1, 370.

Simmons, R. T., and Were, S. O. M. (1955). A "new" family blood group antigen and antibody (By) of rare occurrence. *Med. J. Australia*, ii, 55.

Sneath, Joan S., and Sneath, P. H. A. (1955). Transformation of the Lewis groups of human red cells. *Nature*, 176, 172.

Stratton, F. (1956). Lewis antibodies—with a note on their preservation. Boston: VIth Congress of the International Soc. of Blood Transf.

Stratton, F., and Dimond, E. R. (1955). The value of a serum and albumin mixture for the detection of blood group antigen-antibody reactions. *J. Clin. Path.*, 8, 218.

Stratton, F., and Rawlinson, Violet I. (1958). To be published.

Sussman, L. N., and Miller, E. B. (1952). Un nouveau facteur sanguin "Vel". *Rev. d'Hém.*, 7, 368.

Taylor, G. M., and Prior, Aileen M. (1939). The distribution of the M and N factors in random samples of different races. *Ann. Eugen.*, 9, 97.

Vaughan, J. H., and Waller, Marion V. (1957). Immunologic features of erythrocyte sensitization. II. The nature of blood group antibodies. *Blood*, 12, 29.

Wallace, J., Milne, G. R., Mohn, J., Lambert, R. M., Rosamila, H. G., Moores, Phyllis, Sanger, Ruth, and Race, R. R. (1957). Blood group antigens Mi^a and Vw and their relation to the MNSs system. *Nature*, 179, 478.

Walsh, R. J., [illegible] (1947). A new human isoagglutinin [illegible] *Nature*, 160, 504.

Wie[illegible] Severe erythroblastosis fetalis caused by sensitization to a rare human agglutinogen. *Amer. J. Hum. Genet.*, 5, 350.

Wiener, A. S., Unger, L. J., and Gordon, Eve B. (1953). Fatal hemolytic transfusion reaction caused by sensitization to a new blood factor U. *J. Amer. Med. Ass.*, 153, 1444.

Tables of Blood Group Frequencies

TABLE III. FREQUENCIES OF PHENOTYPES

Bloods of Caucasian origin except where figures for other races are of special interest.

System	Phenotype	Frequency %	Reference
ABO ..	A_1	34·8 } 44·7	Ikin *et al.*, 1939.
	A_2	9·9 }	(These frequencies refer to the South of England.
	O	43·5	Frequencies for the U.K.,
	B	8·6	but without subtypings of
	A_1B	2·6 } 3·2	A, may be obtained from
	A_2B	0·6 }	Dobson and Ikin, 1946)
	A_3	1 in 1,000 of group A	Gammelgaard, 1942.
	A_4	1 in 8,000–1 in 40,000	Dunsford, 1955.
	A_5, A_x, A_0, A_m, A_z	Very rare	
	B_3, B_w, B_x	Very rare	
	O_h	Very rare	Simmons and D' Sena, 1955, Bhatia *et al.*, 1955.
	Secretors	78	Race and Sanger, 1954.
	Non-secretors	22	
Becker ..	Becker positive	0 in 272 tested	Elbel and Prokop, 1951.
Berrens .	Be(a+)	0 in 448 tested	Davidsohn *et al.*, 1953.
Batty ..	By(a+)	0 in 500 tested	Simmons and Were, 1955.
Cavaliere	Ca positive	0 in 48 tested	Wiener and Brancato, 1953.
Chr ..	Chr(a+)	0·4	Kissmeyer-Nielson, 1955.
Diego ..	Di(a+)	0 in 1,000 Whites	Levine and Robinson, 1957.
		2–36, Mongoloids, Negroids, Hybrid Venezuelan populations	Layrisse and Arends, 1957.
Duffy ..	Fy(a+b−)	17·2	Race and Sanger, 1954.
	Fy(a+b+)	48·5	
	Fy(a−b+)	34·3	
	New York Negroes:		
	Fy(a+b−)	8·8	Sanger, Race and Jack, 1956.
	Fy(a+b+)	1·6	
	Fy(a−b+)	21·6	
	Fy(a−b−)	68·0	

TABLE 111.—*contd.*

System	*Phenotype*	*Frequency %*	*Reference*
Graydon	Graydon positive	0 in 61 tested	Graydon, 1946.
Js ..	Js positive	0 in 240 Whites 19 Negroes	Giblet, 1958.
Kell ..	K+k+ K+k− K−k+ K−k−	9·5 0·3 90·2 Very rare	Allen and Lewis, 1957.
	Kp(a+b+) Kp(a+b−) Kp(a−b+) Kp(a−b−)	2·15 About 0·036 97·8 Very rare	Allen (cited by Chown *et al.*), 1957.
Kidd ..	Jk(a+b−) Jk(a+b+) Jk(a−b+)	32·8 48·9 18·3	see p. 302.
Levay ..	Levay positive	0 in 350 tested	Callender and Race, 1946.
Lewis* ..	Le(a+b−) Le(a−b+) Le(a−b−)	22·4 71·6 6·0	Race and Sanger, 1954.
Lutheran	Lu(a+b−) Lu(a−b+) Lu(a+b+)	0·15 92·35 7·5	Race and Sanger, 1954.
MNS	See Table 114 M_1, M^c, N_2	 Very rare	
	S^u positive	100 Whites 98·8 N.Y. Negroes	Wiener *et al.*, 1954
	Henshaw positive	0 in 1,500 Whites 2–14 Africans	Chalmers *et al.*, 1953. Shapiro, 1956.
	Hunter positive	0·5 Whites 7·3 American Negroes 21·7 West Africans	Landsteiner *et al.*, 1934. Mourant *et al.* (cited by Race and Sanger), 1954.
	Mi(a+) Vw positive	0·3 About 0·15	Mohn, 1957. Wallace *et al.*, 1957, Mohn, 1957.
P ..	P positive Tj(a+)	78·9 Almost 100	Race and Sanger, 1954. Levine *et al.*, 1951. Walsh and Kooptzoff, 1954.

APPENDIX

Tables of Blood Group Frequencies

TABLE III. FREQUENCIES OF PHENOTYPES

Bloods of Caucasian origin except where figures for other races are of special interest.

System	*Phenotype*	*Frequency %*	*Reference*
ABO ..	A_1 A_2 O B A_1B A_2B	34·8 } 44·7 9·9 } 43·5 8·6 2·6 } 3·2 0·6 }	Ikin *et al.*, 1939. (These frequencies refer to the South of England. Frequencies for the U.K., but without subtypings of A, may be obtained from Dobson and Ikin, 1946)
	A_3 A_4 A_5, A_x, A_0, A_m, A_g B_3, B_w, B_x O_h	1 in 1,000 of group A 1 in 8,000–1 in 40,000 Very rare Very rare Very rare	Gammelgaard, 1942. Dunsford, 1955. Simmons and D' Sena, 1955, Bhatia *et al.*, 1955.
	Secretors Non-secretors	78 22	Race and Sanger, 1954.
Becker ..	Becker positive	0 in 272 tested	Elbel and Prokop, 1951.
Berrens .	Be(a+)	0 in 448 tested	Davidsohn *et al.*, 1953.
Batty ..	By(a+)	0 in 500 tested	Simmons and Were, 1955.
Cavaliere	Ca positive	0 in 48 tested	Wiener and Brancato, 1953.
Chr ..	Chr(a+)	0·4	Kissmeyer-Nielson, 1955.
Diego ..	Di(a+)	0 in 1,000 Whites 2–36, Mongoloids, Negroids, Hybrid Venezuelan populations	Levine and Robinson, 1957. Layrisse and Arends, 1957.
Duffy ..	Fy(a+b−) Fy(a+b+) Fy(a−b+) New York Negroes: Fy(a+b−) Fy(a+b+) Fy(a−b+) Fy(a−b−)	17·2 48·5 34·3 8·8 1·6 21·6 68·0	Race and Sanger, 1954 Sanger, Race and Jack, 1956.

TABLE 111.—*contd.*

System	*Phenotype*	*Frequency %*	*Reference*
Graydon	Graydon positive	0 in 61 tested	Graydon, 1946.
Js ..	Js positive	0 in 240 Whites 19 Negroes	Giblet, 1958.
Kell ..	K+k+ K+k− K−k+ K−k−	9·5 0·3 90·2 Very rare	Allen and Lewis, 1957.
	Kp(a+b+) Kp(a+b−) Kp(a−b+) Kp(a−b−)	2·15 About 0·036 97·8 Very rare	Allen (cited by Chown *et al.*), 1957.
Kidd ..	Jk(a+b−) Jk(a+b+) Jk(a−b+)	32·8 48·9 18·3	see p. 302.
Levay ..	Levay positive	0 in 350 tested	Callender and Race, 1946.
Lewis* ..	Le(a+b−) Le(a−b+) Le(a−b−)	22·4 71·6 6·0	Race and Sanger, 1954.
Lutheran	Lu(a+b−) Lu(a−b+) Lu(a+b+)	0·15 92·35 7·5	Race and Sanger, 1954.
MNS	See Table 114 M_1, M^c, N_1	 Very rare	
	S^u positive	100 Whites 98 8 N.Y. Negroes	Wiener *et al.*, 1954
	Henshaw positive	0 in 1,500 Whites 2–14 Africans	Chalmers *et al.*, 1953. Shapiro, 1956.
	Hunter positive	0·5 Whites 7·3 American Negroes 21·7 West Africans	Landsteiner *et al.*, 1934. Mourant *et al.* (cited by Race and Sanger), 1954.
	Mi(a+) Vw positive	0·3 About 0·15	Mohn, 1957. Wallace *et al.*, 1957, Mohn, 1957.
P ..	P positive Tj(a+)	78·9 Almost 10[illegible]	[illegible]

TABLE 111—*contd.*

System	*Phenotype*	*Frequency %*	*Reference*
Romunde	R_m positive	0 in 200 tested	van der Hart *et al.*, 1954.
Rh ..	See Table 112		
	E^w positive	0 in 1,299 tested	Greenwalt and Sanger, 1955.
	C^x positive	0·1	Stratton and Renton, 1954.
	C^w positive	2·6	Race and Sanger, 1954.
	f positive	65·7	Race and Sanger, 1954.
	V positive	0 5 Whites 22–40 Negroes	De Natale *et al.*, 1955.
	D^u See Table 113		
Vel ..	Vel positive	99 96	Sussman and Miller, 1952.
Ven ..	Ven positive	0 in 170 tested	van Loghem *et al.*, 1952.
Wright .	Wr(a+)	0 3	van Loghem *et al.*, 1955.
Yt† ..	Yt(a+b−) Yt(a+b+) Yt(a−b+)	88·06 11·56 0·38	Eaton *et al.*, 1956.

* These figures refer to adults, and do not take into account the suppressing effect of A_1. In the Manchester area Le(a−b−) seems to be more frequent than 6 per cent, perhaps 10 per cent or more.

† Phenotypes distinguished by dosage effects.

The list of family antigens may not be exhaustive.

TABLE 112. THE Rh FREQUENCIES
(Figures taken from Race and Sanger, 1954, but anti-C = anti-C+C^w)

	Antisera		*Rh Group*			*Genotype*	
			Frequency				
	C D E	*c e*	*Tested with anti-C, D, E*	*Tested with anti-C, D, E, c, e*	*Group*	*Genotype*	*Frequency*
D Negative	− − −	+ +	15·1020	15·1020	rr	rr	15·1020
	+ − −	+ +	0·7741	0·7644	R′r	R′r	0·7644
		− +		0·0097	R′R′	R′R′	0·0097
	− − +	+ +	0 9376	0 9235	R″r	R″r	0 9235
		+ −		0·0141	R″R″	R″R″	0·0141
	+ − +	+ +	0·0234	0·0234	R′R″	R′R″ $R_y r$	0·0234 0 0000
		+ −		0·0000	R″R_y	R″R_y	0·0000
		− +		0 0000	R′R_y	R′R_y	0·0000
		− −		0·0000	$R_y R_y$	$R_y R_y$	0·0000

TABLE 112—*contd.*

						Genotypes	Frequencies
D Positive	− + −	+ +	2·0609	2·0609	R_0r	R_0r R_0R_0	1·9950 0·0659
	+ + −	+ +	53·3972	34·8899	R_1r	R_1r R_1R_0 $R'R_0$	32·6808 2·1586 0·0505
		− +		18·5073	R_1R_1	R_1R_1 R_1R'	17·6803 0·8270
	− + +	+ +	14·0769	11·7510	R_2r	R_2r R_2R_0 $R''R_0$	10·9657 0·7243 0·0610
		+ −		2·3259	R_2R_2	R_2R_2 R_2R''	1·9906 0·3353
	+ + +	+ +	13·6279	13·3433	R_1R_2	R_1R_2 R_1R'' R_2R' R_zr R_zR_0 R_yR_0	11·8648 0·9992 0·2775 0·1893 0·0125 0·0000
		+ −		0·0745	R_2R_z	R_2R_z $R''R_z$ R_2R_y	0·0687 0·0058 0·0000
		− +		0·2095	R_1R_z	R_1R_z $R'R_z$ R_1R_y	0·2047 0·0048 0·0000
		− −		0·0006	R_zR_z	R_zR_z R_zR_y	0·0006 0·0000
		Totals:	100·0000	100·0000	18 groups	36 genotypes	100·0000

TABLE 113. D^u FREQUENCIES

Apparent group	*Proportion reacting as D^u*
$R'r$	30–45% Approximately
$R''r$	10–20% ”
rr	0·07–0·5% ”
All bloods	0·3–0·6% ”

TABLE 114. THE MNSs FREQUENCIES
Figures from Race and Sanger, 1954

	SS	*Ss*	*ss*	*Totals*
M	6·1	14·0	8·0	28·1
MN	4·0	23·8	22·1	49·9
N	0·6	6·2	15·2	22·0
TOTALS	10·7	44·0	45·3	100·0

TABLE 115. FREQUENCY OF OCCURRENCE OF ANTIBODIES

Regular	Anti-A, B.
Common	Anti-A_1, D, C+D, O, H, P, Le^a, Le^b. Anti-Wr^a, Mi^a, Vw (but the antigens are rare).
Uncommon	Anti-c, c+E, E, D+E, M, S, K, Fy^a, Lu^a. Anti-C+e, e. (Usually in acquired haemolytic anaemia.)
Rare	All others.

TABLE 116. THE BLOOD GROUP ANTIBODIES

Percentage positive results arranged in alphabetical order for bloods of Caucasian origin

Antibody	Percentage positive
Anti-A	47·9
Anti-A_1	37 4
Anti-B	11·8
Anti-Be^a	0 in 448 tested
Anti-Becker	0 in 272 tested
Anti-By^a	0 in 500 tested
Anti-c	81·3
Anti-C	67·8
Anti-C+e	97·7
Anti-C+D	84·0
Anti-c+E	81·5
Anti-Ca	0 in 48 tested
Anti-Chr^a	0·4
Anti-C^w	2·6
Anti-C^x	0·1
Anti-D	83 2
Anti-D+E	84 2
Anti-Di^a	*0 in 1,000 tested*
Anti-E	28 7
Anti-e	97·6
Anti-E^w	0 in 1,299 tested
Anti-f	65·7
Anti-Fy^a	65·7
Anti-Fy^b	82 8
Anti-Gr	0 in 61 tested
Anti-H	Varies with serum & ABO group
Anti-He	0 in 1,500 tested
Anti-Hu	0·5
Anti-Jk^a	81·7
Anti-Jk^b	67·2
Anti-Js	0 in 240 tested
Anti-K	9·8
Anti-k	99·7
Anti-Kp^a	2·2
Anti-Kp^b	99·95
Anti-Le^a	22·4
Anti-Le^b	41·8 (Group A_1)* 71·6 (other ABO groups)
Anti-Le^a+Le^b	94 4 (Group. O)
Anti-Levay	0 in 350 tested
Anti-Lu^a	7·7
Anti-Lu^b	99 85
Anti-M	78·0
Anti-Mi^a	0·3
Anti-N	71·9
Anti-O	*Varies with serum and* ABO group
Anti-P	78·9
Anti-Rm	0 in 200 tested
Anti-S	54 7
Anti-s	89·3
Anti-S^u	100·0
Anti-Tj^a	almost 100
Anti-V	0·5
Anti-Vel	99·96
Anti-Ven	0 in 170 tested
Anti-Vw	0·15 approximately
Anti-Wr^a	0·3
Anti-Yt^a	99 6

* According to Andresen (1948), but varies with different sera.
References as in Table 111.

TABLE 117. THE BLOOD GROUP ANTIBODIES

Percentage positive results arranged in numerical order for bloods of Caucasian origin

Antibody	%	Antibody	%
Anti-Kp^b	over 99*	Anti-Fy^a	65·7
Anti-k	over 99*	Anti-S	54·7
Anti-Lu^b	over 99*	Anti-A	47·9
Anti-S^s	over 99*	Anti-Le^b (Group A_1)	41·8†
Anti-Tj^a	over 99*	Anti-A_1	37·4
Anti-Vel	over 99*	Anti-E	28·7
Anti-Yt^a	over 99*	Anti-Le^a	22·4
Antibodies of -D-/-D-	over 99*	Anti-B	11·8
" " O_h	over 99*	Anti-K	9·8
Anti-H	Variable	Anti-Lu^a	7·7
Anti-O	Variable	Anti-C^w	2·6
Anti-C+e	97·7	Anti-Kp^a	2·2
Anti-e	97·6	Anti-Becker	less than 1*
Anti-Le^a+Le^b (Gp. O)	94·4	Anti-Be^a	less than 1*
Anti-s	89·3	Anti-By^a	less than 1*
Anti-D+E	84·2	Anti-Ca	less than 1*
Anti-C+D	84·0	Anti-Chr^a	less than 1*
Anti-D	83·2	Anti-C^x	less than 1*
Anti-Fy^b	82·8	Anti-Di^a	less than 1*
Anti-Jk^a	81·7	Anti-E^w	less than 1*
Anti-c+E	81·5	Anti-Gr	less than 1*
Anti-c	81·3	Anti-He	less than 1*
Anti-P	78·9	Anti-Hu	less than 1*
Anti-M	78·0	Anti-Js	less than 1*
Anti-N	71·9	Anti-Levay	less than 1*
Anti-Le^b (Gp. O, A_2)	71·6	Anti-Mi^a	less than 1*
Anti-C	67·8	Anti-Rm	less than 1*
Anti-Jk^b	67·2	Anti-V	less than 1*
Anti-f	65·7	Anti-Ven	less than 1*
		Anti-Vw	less than 1*
		Anti-Wr^a	less than 1*

* For further details see Table 111.
† According to Andresen (1948), but varies with different sera.
References as in Table 111.

References

Allen, F. H., and Lewis, Sheila J. (1957). Kp^a (Penny), a new antigen in the Kell blood ... *Sang.*, 2, 81.

Bhat ... G., and Jhala, H. I. (1955). Anti-H ... *J. Ind. Med. Assoc.*, 25, 545.

Call ... (1946). A serological and genetical study of multiple antibodies formed in response to blood transfusion by a patient with lupus erythematosus diffusus. *Ann. Eugen.*, 13, 102.

Chalmers, J. N. M., Ikin, Elizabeth W., and Mourant, A. E. (1953). A study of two unusual blood-group antigens in West Africans. *Brit. Med. J.*, ii, 175.

Chown, B., Lewis, Marion, and Kaita, Kiroko (1957). A "new" Kell blood group phenotype. *Nature*, 180, 711.

Davidsohn, I., Stern, K., Strauser, E. R., and Spurrier, Wilma (1953). Be, a new "private" blood factor. *Blood*, 8, 747.

DeNatale, A., Cahan, A., Jack, J. A., Race, R. R., Sanger, Ruth (1955). V, a "new" Rh antigen, common in Negroes, rare in white people. *J. Amer. Med. Ass.*, 159, 247.

Dobson, Aileen M., and Ikin, Elizabeth (1946). The ABO blood groups in the United Kingdom; frequencies based on a very large sample. *J. Path. Bact.*, 58, 221.

Dunsford, I. (1955). A_4 versus factor C. Preliminary notes. *Vox Sang.*, 5, 104.

Eaton, B. R., Morton, J. A. Pickles, Margaret M., and White, K. E. (1956). A new antibody, anti-Yt^a, characterizing a blood group antigen of high incidence. *Brit. J. Haem.*, 2, 333.

Elbel, H., and Prokop, O. (1951). Ein neues erbliches Antigen als Ursache gehäufter Fehlgeburten. *Zeitschr. F. Hygiene*, 132, 120.

Gammelgaard, A. (1942). Om Sjaeldne, Svage A-receptor (A_3, A_4, A_5, og A_x), Hos Mennesket. Published by Nyt Nordisk Forlag, Arnold Busck, p. 137.

Graydon, J. J. (1946). A rare isohaemagglutinogen. *Med. J. Austral.*, 2, 9.

Greenwalt, T. J., and Sanger, Ruth (1955). The Rh antigen E^v. *Brit. J. Haem.*, 1, 52.

Hart, Mia van der, Bosman, Hélène, and Loghem, J. J. van (1954). Two rare human blood group antigens. *Vox Sang.*, 4, 108.

Ikin, Elizabeth, W., Prior, Aileen M., Race, R. R., and Taylor, G. L. (1939). The distribution of the A_1, A_2, B, O blood groups in England. *Ann. Eugen.*, 9, 409.

Landsteiner, K., Strutton, W. R., and Chase, M. W. (1934). An agglutination reaction observed with some human bloods, chiefly among Negroes. *J. Immunol.*, 27, 469.

Layrisse, M., and Arends, T. (1957). The Diego system—steps in the investigation of a new blood group system. Further studies. *Blood*, 12, 115.

Levine, P., Bobbitt, O. B., Waller, R. K., and Kuhmichel, A. (1951). Immunization by a new blood factor in tumor cells. *Proc. Soc. Exp. Biol. and Med.*, 77, 403.

Levine, P., and Robinson, Elizabeth A. (1957). Some observations on the new human blood factor Di^a. *Blood*, 12, 448.

Loghem, J. J. van, Bok, J., Hart, Mia van der, and Brinkerink, P. C. (1955). Two further examples of the antibody anti-Wr^a (Wright). *Vox Sang.*, 5, 130.

Loghem, J. J. van and Hart, Mia van der (1952). Een zeldzaam voorkomende bloedgroep (Ven). *Bull. van het Centraal Laboratorium van de Bloedtransfusiedienst van het Nederlandse Rode Kruis*, 2, 225.

Mohn, J. F. (1957). Personal communication.

Race, R. R., and Sanger, Ruth. *Blood Groups in Man.* 2nd Ed. 1954. Oxford: Blackwell Scientific Publications.

Sanger, Ruth, Race, R. R., Jack, J. A. (1955). The Duffy blood groups of New York Negroes; the phenotype Le(a—b—). *Brit. J. Haem.*, 1, 370.

Shapiro, M. (1956). Inheritance of the Henshaw (He) blood factor. *Jour. Forensic Med.*, 3, 152.

Simmons, R. T., and D'Sena, G. W. L. (1955). Anti-H in group O blood. *J. Ind. Med. Assoc.*, 24, 325.
Simmons, R. T., and Were, S. O. M. (1955). A "new" family blood group antigen and antibody (By) of rare occurrence. *Med. J. Austr.*, ii, 55.
Stratton, F., and Renton, P. H. (1954). Haemolytic disease of the newborn caused by a new Rh antibody, anti-C^x. *Brit. Med. J.*, i, 962.
Sussman, L. N., and Miller, E. B. (1952). Un nouveau facteur sanguin, "Vel". *Rev. d'Hém.*, 7, 368.
Wallace, J., Milne, G. R., Mohn, J., Lambert, R. M., Rosamilia, H. G., Moores, Phyllis, Sanger, Ruth, and Race, R. R. (1957). Blood group antigens, Mi^a and Vw, and their relation to the MNSs system. *Nature*, 179, 478.
Walsh, R. T., and Kooptzoff, Olga (1954). The human blood group Tj^a. *Aust. J. Exp. Biol. Med. Sci.*, 32, 387.
Wiener, A. S., and Brancato, G. J. (1953). Severe erythroblastosis fetalis caused by sensitization to a rare human agglutinogen. *Amer. J. Hum. Genet.*, 5, 350.
Wiener, A. S. Unger, L. J., and Cohen, Laura (1954). Distribution and heredity of blood factor U. *Science*, 119, 734.

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